Bacterial aggregation by depletion attraction: *Sinorhizobium meliloti* and its extracellular polysaccharide succinoglycan

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Declaration

I hereby declare that the work presented in this thesis is my own except where otherwise stated.

Gary Dorken, September 2009

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Abbreviations

Π	osmotic pressure
°C	degrees celsius
µg	microgram
µl	microlitre
µm	micron/micrometre
×g	acceleration due to gravity
Abs	Absorbance
CFU	colony forming unit
Cm^R	chloramphenicol resistant
Da	dalton
DAPI	4',6-Diamidino-2-phenylindol
dH_2O	deionised water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
g	gram
Gal	galactose
GFP	green fluorescent protein
Glc	glucose
Gm^R	gentamycin resistant
HMW	high molecular weight
in^2	square inch
kDa	kiloDalton
kdo	3-deoxy-D-manno-oct-2-ulusonic acid
Km^R	kanamycin resistant
l	litre
LB	Luria Bertani medium
lb	pounds
LB_{MC}	Luria Bertani medium supplemented with 2.5mM magnesium sulphate and 2.5mM calcium chloride
LMW	low molecular weight

LPS	lipopolysaccharide
M	molar
Mb	megabase
mg	mg
min^{-1}	per minute
ml	millilitres
mM	millimolar
mm	millimetres
MOPS	morpholine propane sulfonic acid
nm	nanometre
Nm^R	neomycin resistant
OD	optical density
OD_{595}	optical density measured at 595nm
OD_{600}	optical density measured at 600nm
PBS	phosphate buffered saline
PHB	polyhydroxybutyrate
PSS	polystyrene sodium sulfonate
r	radius
rpm	revolutions per minute
SD	standard deviation
SE	standard error
SEM	scanning electron microscopy
Sm^R	streptomycin resistant
Sp^R	spectinomycin resistant
Tc^R	tetracycline resistant
TEM	transmission electron microscopy
TLC	thin layer chromatography
Tn	transposon
UDP	uridine diphosphate
UV	ultraviolet
$V_{overlap}$	volume of overlap
w/v	weight per volume

Abstract

In their natural environments microorganisms exist predominantly in aggregates and biofilms. The ability of bacteria to form aggregates is associated with the biosynthesis of polymers such as polysaccharides. In this study the physical mechanisms underlying bacterial aggregation by extracellular polysaccharides are investigated by utilising the bacterium *Sinorhizobium meliloti*. *S. meliloti* biosynthesises an extracellular polysaccharide called succinoglycan, which is well characterised in terms of its structure and biosynthesis. A range of previously constructed succinoglycan biosynthesis mutants were screened for altered aggregation. An *S. meliloti exoS* mutant (a gain of function mutation that results in a constitutively active two component regulator called ExoS) overproduces succinoglycan and has enhanced aggregation compared to the parent strain, Rm1021. The aggregates settle to the bottom of the culture vessel resulting in loss of turbidity of the cultures and phase separation. Microscopic observation showed that succinoglycan did not appear to be attached to the aggregates, which formed ordered structures of laterally aligned cells. By addition of purified succinoglycan it was found that the critical concentration of polymer required to induce aggregation and phase separation of the cultures decreased with increasing cell concentration. These observations suggest that aggregation of *S. meliloti* cultures in the presence of succinoglycan is driven by macromolecular crowding, otherwise known as depletion attraction. Depletion attraction can drive the ordered arrangement and aggregation of colloidal particles in the presence of polymers. Aggregation of the particles increases the volume available to the polymers, maximising their entropy and the entropy of the system. Addition of succinoglycan to stationary phase *Escherichia coli* cultures and polystyrene colloids also resulted in aggregation consistent with depletion attraction. Furthermore alternative polymers such as the bacterial extracellular polysaccharide xanthan produced by *Xanthomonas campestris* can result in aggregation of bacteria by depletion attraction. Depletion attraction may therefore be a ubiquitous force driving aggregation of crowded dispersions of bacteria and polymers.

The second part of the thesis focuses on how depletion driven aggregation can lead to surface-associated biofilm formation. Imaging of the sediment formed by the *exoS* mutant showed that the structure formed at the base of the culture vessel leads to development of an ordered structure composed of interlinked aggregates. The role of succinoglycan in surface attachment is complex and varies with culture conditions. Depletion attraction may facilitate interaction with a surface but alternative factors may then play a role in anchoring the cells to the surface. Under certain conditions the cells may produce factors which allow binding of the cells to a surface independently of succinoglycan.

This study has demonstrated for the first time that an extracellular polysaccharide produced by bacteria can result in aggregation via depletion attraction which may be an under explored mechanism by which aggregation of bacteria can occur.

Chapter 1

Introduction

As well as living as unicellular organisms, bacteria can exist in multicellular aggregates. Polymers, usually in the form of extracellular polysaccharides produced by the bacteria are integral to the formation of bacterial aggregates. The focus of this thesis is the mechanisms underlying polymer-driven bacterial aggregation. Specifically this thesis will be studying the mechanism of aggregation in *Sinorhizobium meliloti* by an extracellular polysaccharide it produces called succinoglycan.

1.1 Aggregates

The growth of bacteria as dispersed cells in pure culture is central to the study of microbiology. However it has become increasingly apparent that this mode of growth may not be prevalent in the natural environment, with bacteria instead commonly forming aggregations of cells [1].

1.1.1. Wastewater treatment and aggregate formation

One area where bacterial aggregation is of interest, and in contrast to other areas of microbiology where enhancement of aggregation is actually desired, is in the study of the activated sludge process of wastewater treatment. The use of microorganisms in the treatment of sewage was developed in 1910-1914 in Manchester and other European cities in response to the increase in the population of cities brought about by the industrial revolution and to ease pollution of waterways [2]. The development of this important biotechnological process may have done as much

to reduce disease as the introduction of antibiotics [2]. Wastewater treatment brings microorganisms into contact with the wastewater to metabolise and break down the organic component of sewage to allow the return of the processed water to natural waterways without causing pollution. The design of wastewater treatment plants can vary but most consist of initial sedimentation and filtration to remove large suspended solids and grit, followed by contact of the waste with microorganisms which metabolise the organic matter of the sewage into biomass or new cells. The final removal of the organic matter then requires sedimentation of the bacteria, so the effluent is run into a settling tank where the cells and biomass are allowed to settle out. The clarified effluent, now virtually free of solids and organic compounds, can at this stage be run into rivers or streams, although further treatment may be necessary. The activated sludge formed from the microorganisms after metabolising the organic compounds in the wastewater can then be collected and disposed of or recycled for the next round of treatment (Fig. 1-1). An important part of the process of wastewater treatment by activated sludge is therefore sedimentation of the bacteria and associated biomass. For this to occur aggregation of the bacteria is essential to ensure efficient and rapid sedimentation of the bacteria and thus the oxidised organic material is fully removed from the treated water. Poor settling of the activated sludge can result in ecological problems due to the release of suspended solids into the environment, along with operating problems for the plant itself [3].

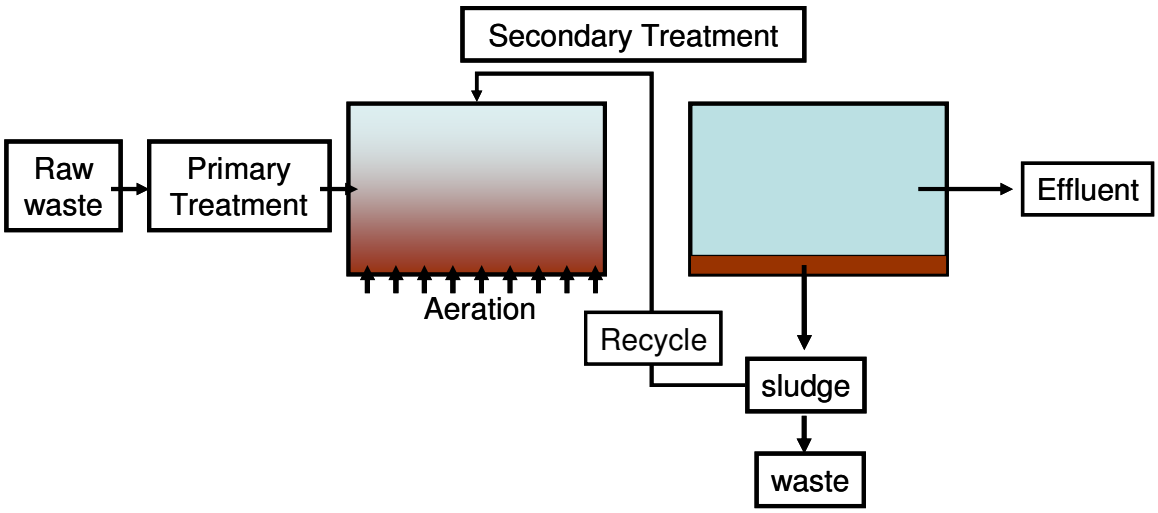


Figure 1-1. Diagram of a simplified wastewater treatment process using the activated sludge process.

Research has therefore been dedicated to understanding and promoting the process of bacterial aggregation to increase the efficiency of wastewater treatment processes. The size of the aggregates formed can range from 20-3000 µm [3]. The aggregates are a highly complex conglomeration of cells and extraneous material, which makes microscopic analysis of the structures extremely difficult [4]. It became clear that the treatment process was a balance between providing aeration and maximal growth conditions of the bacteria to enhance metabolisation of the organics, and ensuring that the cells entered stationary phase which appeared to enhance aggregation [5].

A number of theories were proposed to explain the means by which bacteria aggregate. Initially there was a suggestion that bacterial dispersions were stable due to electrostatic repulsion between cells, with a negative charge of the cell surface being demonstrated by movement of bacteria towards an anode when placed in an electrical field [6]. However cells at their isoelectric point do not aggregate, suggesting that surface charge has only a minor role to play in the stability of dispersions of

cells [7, 8]. Another theory was connected with the observed increase in flocculation when the cells entered stationary phase which was accompanied in some species by an increase in intracellular accumulation of polyhydroxybutyrate (PHB) [8]. However it is generally believed that PHB is an energy reserve for many species of bacteria and is not involved in aggregation [8].

The dominant theory to explain the observed flocculation therefore quickly became focussed on extracellular polymeric material that is accumulated by the bacteria and the resulting aggregates. Observation by electron microscopy showed that the cells in activated sludge were surrounded and entangled in fibrillar material [9-11]. Observation with dyes and fluorescent dyes specific for polysaccharides also showed the presence of a matrix surrounding the cells [4, 11-13]. It was first hypothesised that the production of an extracellular matrix holding the aggregates together was due to the presence in the bacterial community of certain specific species such as *Zoogloea ramigera* [8, 10]. This species is known and characterised by its presence in activated sludge and its ability to produce copious amounts of a gel-like matrix encapsulating the cells, with the name *Zoogloea* derived from Greek and meaning 'living glue' [10]. However subsequent studies working with activated sludge from multiple treatment plants found that several different strains of bacteria could be isolated and formed flocs independently of the presence of *Zoogloea ramigera* [9-11, 13]. The matrix formed by *Zoogloea* species was sensitive to treatment with cellulase, and the presence of slime as a result of polysaccharide production associated with the independently identified aggregate-formers from activated sludge suggested that polysaccharides produced by the cells was a common theme in bacterial aggregation of activated sludge [8-11, 13].

1.1.2. Aggregate formation is associated with extracellular polymers in many species of bacteria

The formation of aggregates by bacteria due to the release of extracellular polymers was realised not just to be a unique property of organisms involved in wastewater processing, but rather a component of the lifestyle of many bacteria. Deinama and Zevenhuizen (1971) [9], initially looking at activated sludge, found that the large aggregates formed by the bacteria were entangled in fibrils, as shown by electron microscopy. Treatment of these species cultures with cellulase resulted in the production of a large quantity of reducing sugars, with thin layer chromatography (TLC) finding only the presence of glucose, suggesting that the polymeric material associated with the flocs was cellulose [9]. As part of this study the authors also examined the aggregate-forming ability of some other species studied in their laboratory; mainly strains of *Rhizobium*, *Sinorhizobium* (formerly *Rhizobium*) and *Agrobacterium* [9]. As they had found with the activated sludge bacteria, strains capable of aggregation appeared to be producing cellulose fibrils (Fig. 1-2) [9]. This study had therefore identified bacteria not involved in activated sludge processes which were also capable of aggregation and had shown that extracellular polymers, in this case cellulose, were involved in the aggregation process.

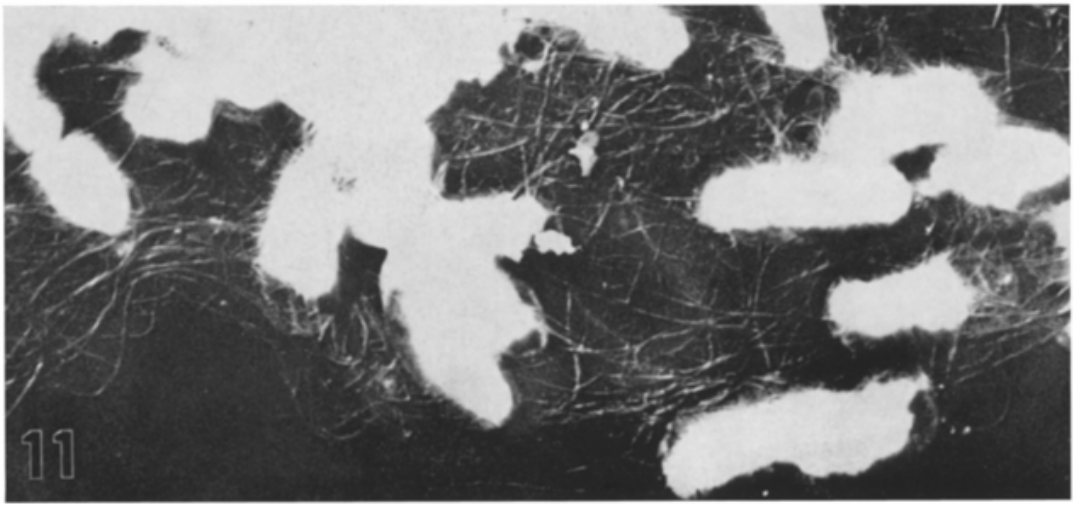


Figure 1-2. Electron microscopy image showing cells of *Agrobacterium* aggregating in the presence of extracellular fibrils, believed to be cellulose [9]

Rhizobium and *Sinorhizobium* species both have agricultural importance as they infect leguminous plants and fix nitrogen from the atmosphere, which is then made use of by the plant. *Agrobacterium* is a plant pathogen, infecting wounds on plants and causing crown gall formation. Other examples of bacteria present in the soil or that interact with plants have been found to form aggregates, and extracellular polysaccharides produced by these bacteria are essential for aggregate formation. Various strains of *Rhizobium leguminosarum* were found to form large flocs which were dependent on synthesis of cellulose fibrils for the formation of aggregates of cells attached to root tips [14]. Cellulose production mutants were constructed and showed that overproduction of cellulose by the cells could enhance aggregation in some strains, and loss of cellulose production led to the loss of aggregation in others (Fig. 1-3) [15]. Similarly in *Agrobacterium* it was found that a cellulose deficient mutant was unable to aggregate and attach to carrot cells in cell culture [16, 17]. As had been found in *R. leguminosarum*, overproduction of cellulose increased the formation of aggregates interacting with roots [18]. In both cases it appears that

production of an extracellular polysaccharide by the bacteria is essential for attachment and aggregation of the cells in large enough numbers to ensure a successful symbiotic or pathogenic infection. *Azospirrilum* species, like *Rhizobium* species, are also capable of fixing nitrogen and inhabit soils around plant roots but do not infect plants. Nevertheless the nitrogen fixing ability of these organisms is thought to promote plant growth indirectly [19]. As with the previous examples the bacteria have been shown to form large aggregates *in vitro*, which is associated with the presence of fibrils and the ability to bind dye specific to β-1,4-glucan linkages (Fig. 1-4) [19, 20]. Moreover the cells within aggregates could survive desiccation for up to six months [19]. *Xanthomonas campestris* is a plant pathogen responsible for causing black rot of cruciferous plants. It is a vascular pathogen, normally restricted to the xylem of the leaves of plants at the early stages of disease. This organism is capable of producing an extracellular polysaccharide called xanthan that is used extensively in various biotechnological processes (see below). While the polysaccharide produced by this organism has been well characterised, few studies have been conducted to ascertain the role the polysaccharide may have in aggregation. While in the parent strain cells grow as a dispersed culture, especially in rich media, alteration of media or certain mutations are able to induce aggregation, which was shown to be associated with xanthan production as a strain unable to biosynthesise xanthan could not form aggregates [21, 22].

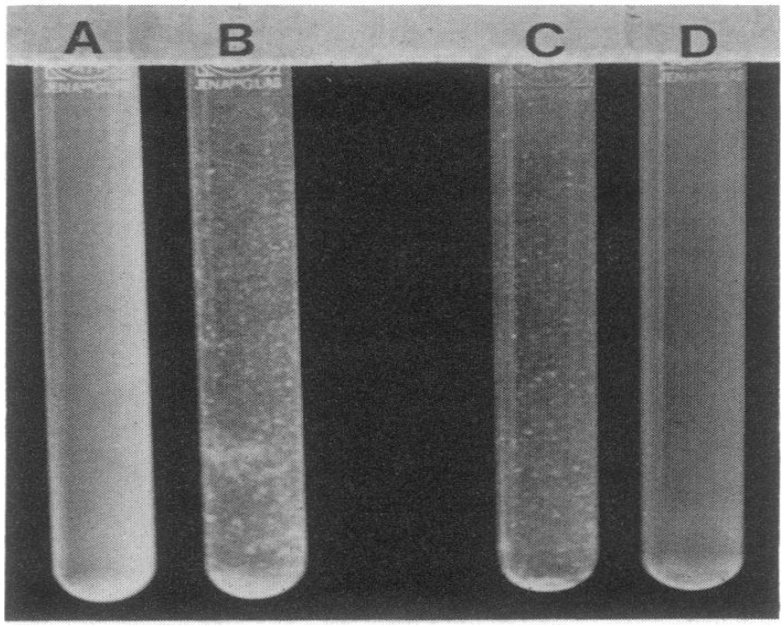


Figure 1-3. Formation of aggregates in *R. leguminosarum* due to production of cellulose after growth in test tubes. A. *R. leguminosarum* strain 248 and B. a cellulose overproducing mutant of this strain. C *R. leguminosarum* strain RBL5523 naturally produces aggregates in culture, which are absent in a mutant deficient in cellulose production (D) [15].

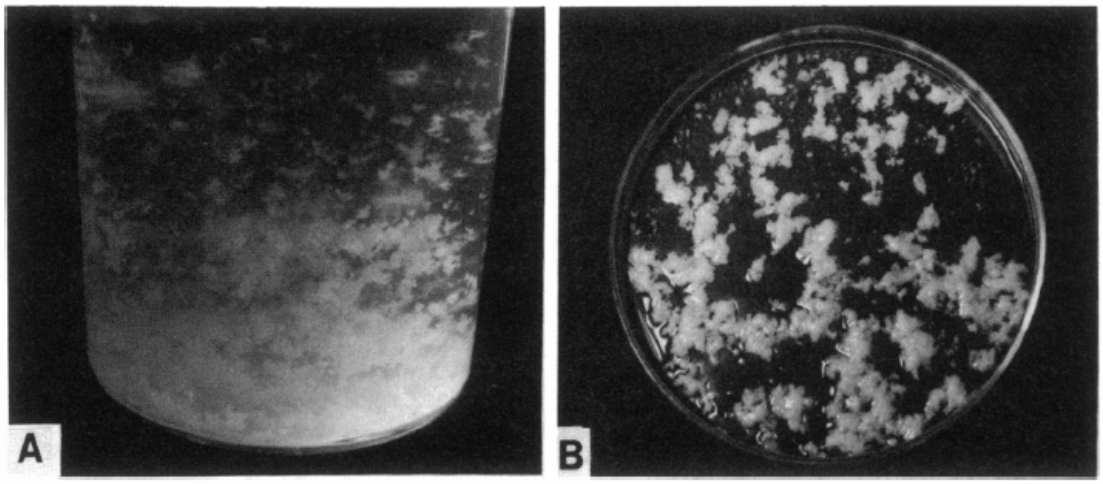


Figure 1-4. Formation of aggregates in *Azospirrilum*. Aggregates were associated with protection from desiccation stress [20].

A final example of the correlation between aggregation and polysaccharide production is provided by *Pseudomonas* species. *Pseudomonas* species are found as a component of activated sludge and are capable of forming aggregates [9]. Further to this *Pseudomonas syringae* was shown to survive desiccation stress on the leaves of

plants by virtue of the formation of aggregates, which the authors partly attributed to production of extracellular polysaccharides [23]. *Pseudomonas aeruginosa* is an opportunistic species, capable of existing in a range of habitats from soil to animals. It is also an opportunistic pathogen and often has a prominent role in the infections of immunocompromised patients such as those suffering from cystic fibrosis. Cystic fibrosis is caused by mutations in the CFTR gene (cystic fibrosis transmembrane regulator, which encodes for a transmembrane chloride ion channel protein), with defective chloride ion channel transport leading to a build up of sticky, dehydrated mucus in the airways of the lung, which leads to fatal bacterial infection [24]. In patients suffering from cystic fibrosis, chronic bacterial infection of the lung with recurrent rounds of infection, often over 10-20 years, is now the leading cause of mortality [24]. The onset of chronic infection, with the bacteria generally becoming resistant to antibiotics is coincident with the cells switching to a mucoid phenotype, attributable to an exopolysaccharide produced by the bacteria called alginate (see below) [24]. Concurrent with this is the observation of clusters of cells encased in a dense extracellular matrix obtained from the sputum of cystic fibrosis patients [25].

The examples of bacterial aggregation given above demonstrate that bacterial aggregation occurs in multiple environments and is associated with the presence of extracellular polysaccharides produced by the bacteria. It should be noted however that other polymers have been detected in the presence of bacterial aggregates that could have a role in aggregation such as nucleic acids, lipids and proteins [3, 7]. In many of the cases stated above the aggregation has been correlated to an ecological role; the increase in the ability to interact with host, resistance to desiccation or a switch to antibiotic resistance and persistence in disease [14, 23, 25, 26].

Synonymous with bacterial aggregation is the idea that bacteria can also colonise and aggregate on surfaces. Such structures are usually referred to as biofilms. These surface-associated structures are often differentiated from bacterial aggregates by the fact that they form on a surface, although recent definitions of biofilms have been extended to include the aggregates referred to above [27]. In the case of biofilm formation the prevailing model is that individual bacteria attach to a surface, followed by the accumulation and aggregation of the cells into a developed 3D structure. Various groups have attempted to characterise the factors important in these processes. Increasingly a molecular approach has been used as the genomes of more microorganisms have become sequenced allowing the identification of mutants deficient in either surface colonisation or maturation of a 3D aggregated biofilm structure [28-30]. Biofilm studies with *Escherichia coli, Vibrio cholerae and P. aeruginosa* suggest that there are common mechanisms of surface colonisation and resulting development of aggregates [28] (Fig. 1-5). Once again in each of these cases extracellular polysaccharides have been shown to be essential, especially with respect to the formation of the cell-cell aggregations that characterise a biofilm [28, 31]. In this thesis a distinction will be made between the formation of cell-cell aggregates and surface colonisation in the form of biofilm formation. Aggregates are referred to as formation of cell-cell clusters in the bulk phase. Biofilms are restricted to discussion of the interaction of bacteria or aggregates with a surface.

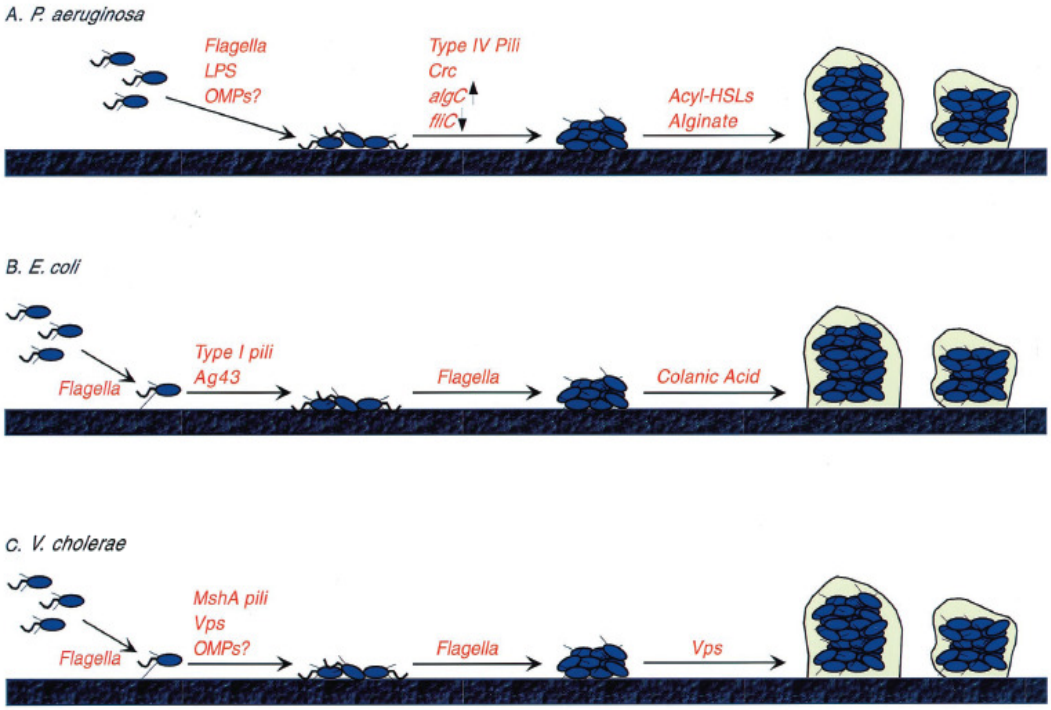


Figure 1-5. Model of biofilm formation by A. *P. aeruginosa*, B. *E. coli* and C. *V. cholerae*. Mutants are screened for biofilm deficiency allowing the factors necessary for surface attachment to be elucidated [28].

1.2 Bacterial extracellular polysaccharides

As shown in the examples above extracellular polysaccharides are therefore integral to aggregation. The bacterial cell surface is the source of a diverse range of polysaccharides. In bacteria the cytoplasmic membrane is surrounded by a cell wall composed of peptidoglycan, which is made up of polysaccharide chains (consisting of N-acetylglucosamine and N-acetylmuramic acid) cross-linked by various amino acids [32]. In Gram-positive bacteria the peptidoglycan is a thick layer, which may also be interspersed with teichoic acid [32]. In Gram-negative bacteria, such as *S. meliloti* which is the focus of this thesis, the peptidoglycan is thinner and situated in the periplasm, which is surrounded by an additional layer, the outer membrane [32]. The

outer membrane is not simply a second lipid bilayer but is composed of various polysaccharides to make a lipopolysaccharide complex (LPS) on the outer leaflet of the membrane (Fig 1-6) [32]. The LPS is composed of lipid A (fatty acid lipids linked to a disaccharide), connected via the disaccharide unit of the lipid A to the core polysaccharide, which in turn can be connected to an O-antigen polysaccharide portion [33] (Fig. 1-6). The polysaccharide composition of the different parts of the LPS varies between species, and different species may not have the full LPS, with some species and strains of bacteria lacking the O-antigen portion.

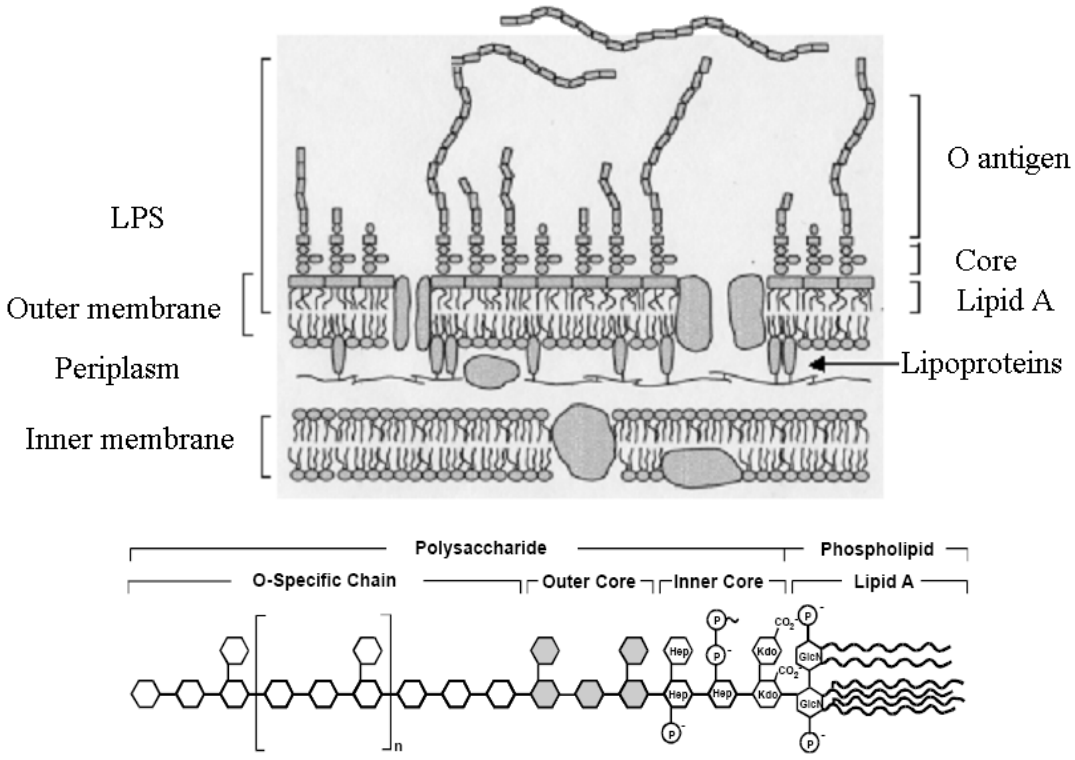


Figure 1-6. Structure of the LPS of Gram negative bacteria. Upper panel shows the structure of the Gram negative cell membrane, including the inner cytoplasmic membrane, periplasm containing peptidoglycan and finally the outer membrane, which is made up of LPS. Lower panel indicates detailed structure of LPS. Some strains may lack the O-antigen substitution [34].

In addition to the polysaccharides that make up the components of the outer membrane there are also extracellular polysaccharides, which can have varying degrees of attachment to the cell surface and are thought to play a dominant role in aggregation as described in the previous section. Extracellular polysaccharides occur in two basic forms. Capsule polysaccharide (capsular polysaccharide, CPS or K-antigen) is closely associated with the cell surface and may be covalently bound to either phospholipid or lipid A molecules [35, 36]. High molecular weight capsules, such as group I K antigens from *E. coli*, give rise to the thick capsules that surround cells and can be visualised by electron microscopy (Fig. 1-7). In contrast exopolysaccharides (slime polysaccharide) lack any form of linker to the cell surface and as such are either loosely associated or totally dissociated from the cell surface and secreted into the growth medium [35, 37]. Exopolysaccharides or slime polysaccharides are responsible for the mucoid appearance of some bacterial colonies when grown on solid agar plates [37]. In technical terms the two can be differentiated by the fact that capsular polysaccharides will remain associated with the cells after centrifugation whereas exopolysaccharides will be washed from the cells into the supernatant [35]. However these definitions do have to be treated with caution and differentiation of the different polysaccharides of the bacterial surface can be difficult. For example some cells can produce an excess of capsular polysaccharide which can then slough from the surface of the cell and be mistaken for exopolysaccharide [36]. Due to the close association of the capsule with the outer membrane, capsular polysaccharide can also be mistaken for LPS [35]. Both capsules and exopolysaccharides have been shown to play a role in the aggregation of cells. For example, observations of sludge aggregates suggested that the cells were joining

at the capsules [4] and the cellulose fibrils produced by *Agrobacterium* and *R. leguminosarum* are secreted into the medium as exopolysaccharides [14-16].

Figure 1-7. Electron microscopy of the *E. coli* group I K antigen. The capsule was stabilised with antibody before the image was taken. Group I K antigens are typically high molecular weight and as such form densely stained thick layers around the cell [35].

The importance of many extracellular polysaccharides in bacterial physiology has been overlooked because in the context of pure culture in the laboratory they appear to be non-essential for bacterial growth [38]. In fact under *in vitro* conditions and successive pure culture methods bacteria may even lose the ability to produce these polysaccharides, a fact which has been realised only as microbiologists have become more interested in the importance of these polymers in aggregate formation [38].

1.2.1 General features of extracellular polysaccharides composition and synthesis

Extracellular polysaccharides are extremely diverse in terms of structure. Primarily composed of monosaccharides linked by glycosidic linkages, they can also contain various non-carbohydrate organic and inorganic substituents [37]. The monosaccharides can be joined in a number of configurations due to the multiple hydroxyl groups that can participate in glycosidic bond formation, which can give rise to branching structures [36]. They can be homopolysaccharides consisting of a single type of monosaccharide such as cellulose, or heteropolysaccharides made up of two or more kinds of monosaccharide such as succinoglycan (see below) [39]. Many polysaccharides are polymers of regular repeating structures, such as succinoglycan and xanthan [35]. Extracellular polysaccharides, in contrast to proteins which are biosynthesised under direct genetic control, are typically polydisperse with molecular weight, and may even differ in composition (for example in the addition of substituents) between each molecule [39]. The polysaccharides can be charged, with many having a polyanionic character, due either to the carbohydrates in their structure (such as uronic acids) or due to non-carbohydrate substitutions (Table 1-1) [37]. It appears that in all cases studied so far, the polysaccharides present in aggregates and biofilms are the same as the polysaccharides produced in pure culture, meaning it is unlikely that bacteria produce a polysaccharide specifically for aggregation [40]. Additionally it is known that many bacterial species can produce multiple capsular and extracellular polysaccharides at the same time, depending on environmental conditions [35, 36, 41].

The organic and inorganic substitutions can alter the properties of the polymer (Table 1-1). Commonly found in bacterial polysaccharides are ester linked acetate (precursor acetyl CoA), which carries no charge, and ketal linked pyruvate (precursor phosphoenolpyruvate), which can contribute to the polyanionic nature of the molecule. The acyl content of some polysaccharides can be quite high, which can increase the lipophilicity of the molecule and affect its ability to interact with other polysaccharides and cations [42]. Inorganic substituents such as sulphate and phosphate have also been found in bacterial polysaccharides.

Table 1-1. Non-carbohydrate substitutions found in bacterial extracellular polysaccharides

Substituent	**Linkage**	**Charge conferred**	**Occurrence**
Organic acids			
Acetate	Ester	None	Very common i.e. *X. campestris, S. meliloti*
Hydroxybutanoate	Ester	None	e.g. *R. leguminosarum*
Pyruvate	Ketal	Negative	Very common i.e. *X. campestris, S. meliloti*
Succinate	Half Ester	Negative	*S. meliloti*
Inorganic acids			
Phosphate		Negative	Common in gram positive bacteria
Sulphate		Negative	Cyanobacteria

In many bacteria the polysaccharides associated with the cell surface; peptidoglycan, LPS, O-antigen as well as the capsular and exopolysaccharides, are all synthesised on a lipid carrier within the cytoplasm before being exported across the membrane. Undecaprenol phosphate (bactorprenol or isoprenoid lipid) has the dual role of providing an anchor on which to polymerise the polysaccharide and

aiding its passage in an activated form [43] (Fig. 1-8). Due to their central role in polysaccharide formation in bacteria the total isoprenoid available may be up to 5.5×10^4 molecules per cell, equivalent to 0.02% of the cells dry mass [37].

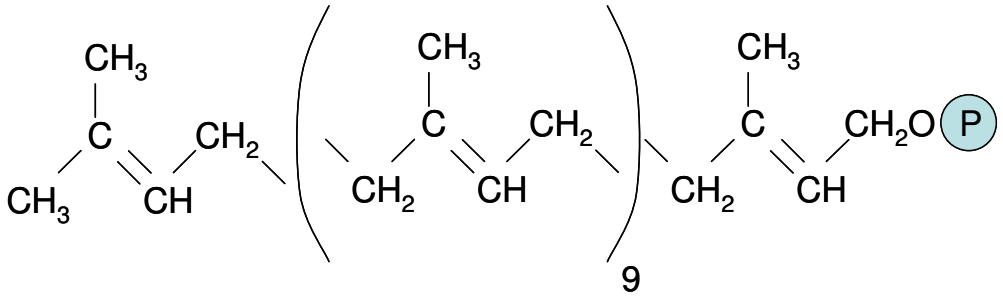


Figure 1-8. Undecaprenol phosphate (bactoprenol or isoprenoid lipid) structure. Used by many bacteria as an anchor on which to assemble polysaccharides such as extracellular polysaccharides or LPS [37].

Polysaccharides are also able to adopt ordered secondary conformations. Flexibility about the glycosidic oxygen between monosaccharides will result in the chain fluctuating due to thermal collisions and as a result there will be an entropic drive for the polymer to take on a random coil conformation [44, 45]. However the polymeric nature of the molecule also means that there will be favourable, cooperative interactions (hydrogen bonding, van der Waals forces) that can fix the molecule into a shape stable enough to overcome the randomizing effect of thermal collisions [44]. The periodic nature of many polysaccharides means that a large number of such favourable interactions can occur between repeating units on the chain, which as well as stabilising the structure can also give rise to a periodic shape such as a helix [44]. Adoption of such structures can change the properties of the polymer. For example formation of an ordered secondary structure such as a helix means that

many polysaccharides become stiffer and rod-like compared to the random coil conformation [42].

1.2.2 Examples of extracellular polysaccharides in bacteria

The production of alginate by *P. aeruginosa* is of considerable interest due mainly to the role of this organism in the fatality of cystic fibrosis patients, as highlighted previously [24]. While cystic fibrosis patients can suffer from recurrent *P. aeruginosa* infections from an early age, a switch in *P. aeruginosa* to a mucoid state is linked to a worsening prognosis for the patient due to the onset of chronic infection with reduced effectiveness of antibiotics [24, 26, 46]. Associated with an increase in mucoidy is an increase in the formation of aggregates or clusters obtained in sputum, surrounded by extracellular material [25, 47] (Fig. 1-9). Alginate is unique compared to other extracellular polysaccharides that have thus far been characterised [42]. Alginate is an extracellular polysaccharide that challenges the definition of capsular vs. exopolysaccharides. Some refer to the alginate produced by *P. aeruginosa* as a capsule [48] or "capsule-like" [49], which is supported by the dense matrix formed around aggregates obtained from the sputum of cystic fibrosis patients (Fig. 1-10). However the copious overproduction of alginate by *P. aeruginosa* when in the mucoid state means that large amounts of polymer are also released into the growth medium and so alginate can also be referred to as a slime polysaccharide or exopolysaccharide [42, 50]. Alginate is a linear copolymer of (β-1- 4) linked β-D-mannuronic acid and its C5 epimer α-L-guluronic acid (Fig. 1-10) [50]. *P. aeruginosa* alginate is not made up of a regular repeating structure; instead it is a highly acetylated polymer composed mainly of mannuronic acid residues interspersed with single guluronic acid residues [51, 52]. *P. aeruginosa* alginate is also exceptional in that it is synthesised in its final

form by a process of post-polymerisation epimerisation, whereby the polymer is initially synthesised as a homopolymer of mannuronic acid, before conversion of some of these residues to its C5 epimer guluronic acid [52]. O-acetyl groups appear to give a protective effect inhibiting epimerisation and giving rise to the large number of mannuronic acid residues in the highly acetylated polymer of *P. aeruginosa* [52]. No undecaprenol-associated intermediate has been identified, suggesting that polymerisation occurs without this lipid intermediate [53].

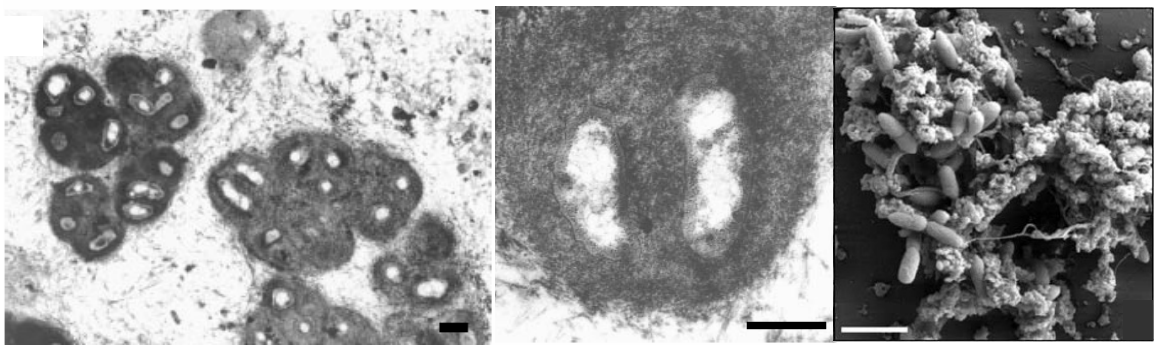


Figure 1-9. TEM electron micrographs of *P. aeruginosa* clusters obtained from the sputum of cystic fibrosis patients [25, 47]. Scale bars: TEM images on left are 1μm, SEM image on the right is 2μm.

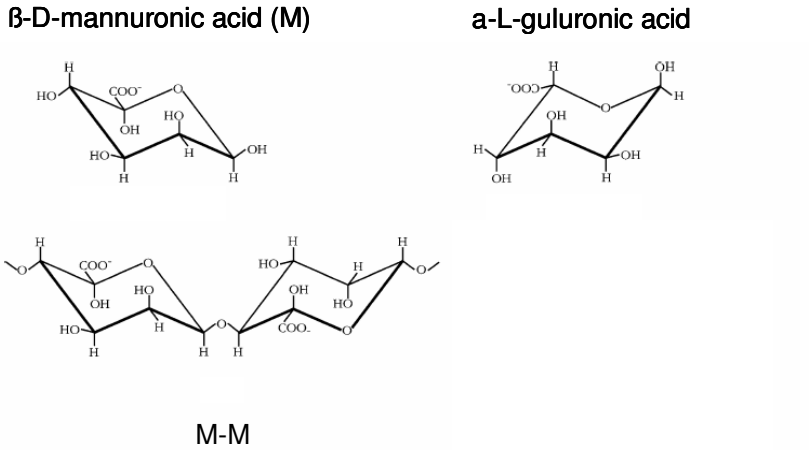


Figure 1-10. Alginate is a polysaccharide composed of mannuronic acid and guluronic acid residues. In *P. aeruginosa* the high amount of acetylation limits the number of guluronic acid residues in the polymer. This means that the polymer is mainly composed of mannuronic acid 'blocks' [53].

While alginate has been seen as the major polysaccharide in biofilm formation of *P. aeruginosa*, recent studies have indicated that non-mucoid *P. aeruginosa* can also form aggregates and produce other polysaccharides which may be playing a role in this aggregation [48, 54]. Two operons were discovered, inactivation of certain genes in either resulting in loss of polysaccharide production and decrease in aggregation [48]. The polysaccharides produced have not been characterised [48].

Xanthan is a well characterised exopolysaccharide produced by *X. campestris*, but until recently its role in aggregation had not been studied. Xanthan has important industrial applications as a thickener and emulsifier in a range of products including foods [55]. The polymer is secreted into the medium, making it an exopolysaccharide, and may cause an increase in viscosity of the medium which can impact on growth of the cells by reducing the ability to aerate the culture by shaking, reducing oxygen levels [40]. Xanthan is an acidic polymer composed of pentasaccharide repeating subunits. It consists of a cellulose backbone, with trisaccharide side chains composed of mannose(β-1,4)glucuronic acid(β-1,2)mannose attached to alternate glucose residues on the backbone by α-1,3 linkages (Fig. 1-11) [56]. On approximately half of the terminal mannose residues a pyruvic acid moiety is joined by a ketal linkage [56]. Acetyl groups are also present as 6-O substituents on internal mannose residues [56]. Xanthan is polymerised on undecaprenol lipid intermediates before secretion from the cell as an exopolysaccharide [37]. Xanthan molecules behave as stiff rods due to formation of ordered helical conformations [42].

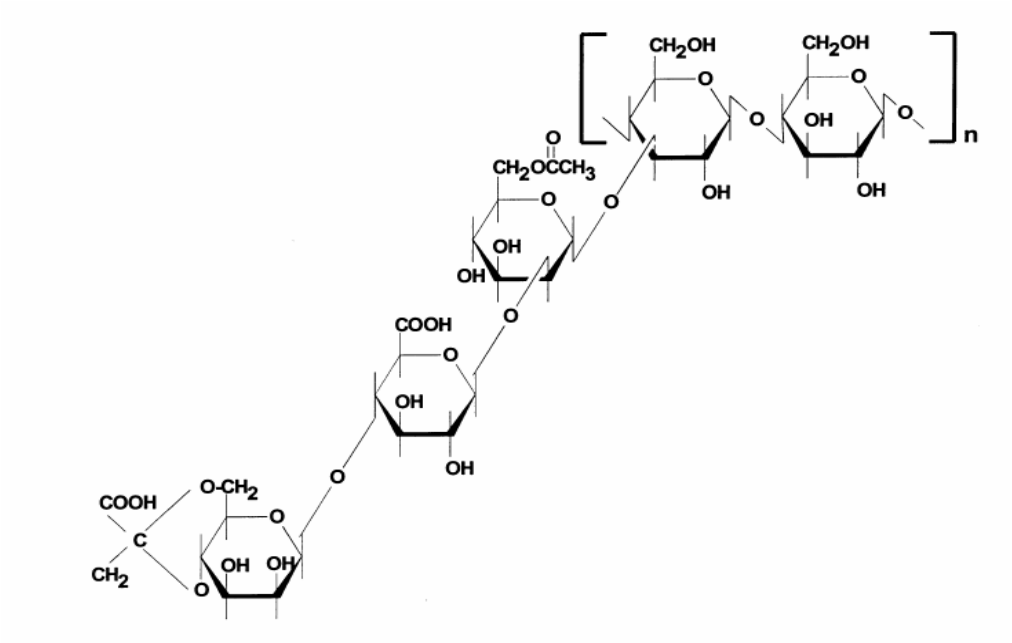


Figure 1-11. Structure of the repeating unit of xanthan [55].

Xanthan and its role in aggregation of *X. campestris* cells have only recently been studied. Dow *et al* (2003) found that when grown in rich media certain mutants formed extensive aggregates rather than growing as dispersed cells as seen in the parent strain [21]. Imaging of these aggregates found that the cells were apparently enclosed in an extensive extracellular matrix, formation of which was dependent on xanthan production (Fig. 1-12). The mutants were found to be defective in production of a predicted extracellular glycanase (endo-β-1,4-mannosidase) [21]. Interestingly despite the apparent need for this enzyme to disperse the aggregates, it was found not to act on xanthan, suggesting that the production of another extracellular polysaccharide was also necessary for aggregate formation.

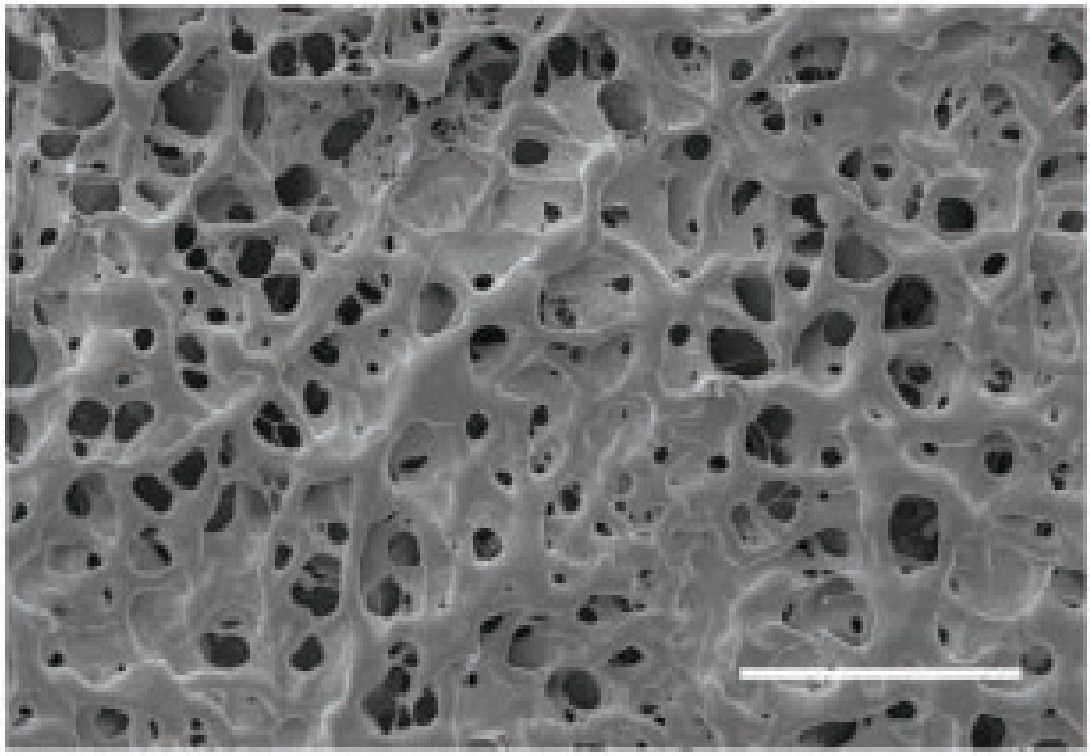


Figure 1-12. Electron micrograph of the aggregates formed by the mutants of *X. campestris*. Mutants did not produce an extracellular glycanase needed for cell dispersal from the aggregates. Bar=10µm [21].

While extracellular polysaccharide production has been well studied in both *P. aeruginosa* and *X. campestris* uncertainties do remain over the role of these polysaccharides in aggregation. It is also unclear whether the strains are capable of producing more than one type of polysaccharide that can be involved in aggregation. Attention will now focus on the subject species of this thesis, *Sinorhizobium meliloti*, strain Rm1021. As will be explained below *S. meliloti* has well characterised extracellular polysaccharide synthesis that makes it useful for studying the effects of extracellular polysaccharides on aggregation.

1.3 *Sinorhizobium meliloti*

S. meliloti (formerly *Rhizobium meliloti*) is a soil bacterium that forms a symbiotic association with leguminous plants such as alfalfa (*Medicago sativa*) fixing nitrogen from the atmosphere into ammonium. The bacteria reside in the rhizosphere, a 1500 µm thick region around the roots of a plant [57]. The symbiosis is initiated by the plant host releasing flavanoids which act as a signal to the bacteria to start infecting the plants. As part of this response the bacteria release lipochitooligosaccharide Nod factors, which when received by the host plant induce multiple responses to allow invasion of the bacteria. Part of this response is deformation of the root hair cells where the bacteria accumulate and eventually invade via a plant-derived infection thread (Fig 1-13). Bacteria divide within the growing infection thread and eventually are released into the cortex cells of the root, where they are enclosed in a membrane and differentiate into bacteroids to begin fixing nitrogen for the plant (Fig. 1-13). The cortical cells holding the bacteroids differentiate into a growth on the root called a nodule. As well as Nod factors a second signal is needed from the bacteria to initiate infection thread development: extracellular polysaccharides.

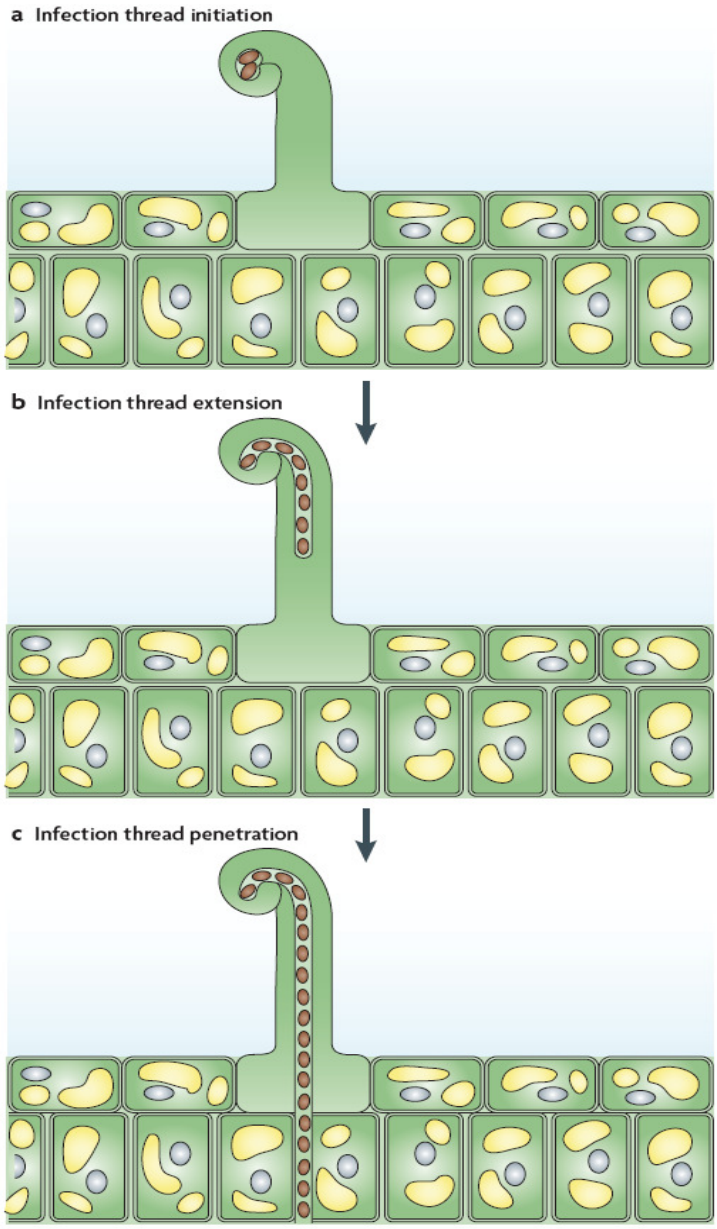


Figure 1-13. Infection thread formation and invasion by *S. meliloti. S. meliloti* occupy deformed root hairs and are able to invade via a plant derived infection thread. For this to occur *S. meliloti* needs to be producing symbiotically active polysaccharides which act as a signal to initiate infection thread formation. Bacteria invade the growing infection thread and multiply, before being released into cortex cells, which differentiate into a nodule [58].

1.3.1 S. meliloti *extracellular polysaccharides*

The importance of extracellular polysaccharides in the initiation of symbiosis was first recognised when inoculation of plants with exopolysaccharide deficient mutants of *S. meliloti* resulted in empty nodules due to defective infection thread development [59, 60]. Use of transposon mutagenesis to disrupt the genes of the *S. meliloti* genome has allowed the isolation and characterisation of a range of exopolysaccharide biosynthesis mutants which has further allowed elucidation of the biosynthetic pathway.

The strain used in this characterisation is *S. meliloti* Rm1021, a spontaneous streptomycin-resistant derivative of SU47, the original environmental isolate of *S. meliloti* [61]. Rm1021 has also had its genome sequenced and has been found to have a 6.7 megabase (Mb) tripartite genome consisting of the chromosome (3.65 Mb) and two megaplasmids, pSymA (1.35 Mb) and pSymB (1.68 Mb) [62]. *S. meliloti* is a Gram negative bacterium. Its outer surface therefore consists of LPS. The lipid A of *S. meliloti* Rm1021 has been characterised and consists of a glucosamine disaccharide linked via the 6' carbon to Kdo (3-Deoxy-D-manno-oct-2-ulosonic acid) in the LPS core [33]. The disaccharide can also be phosphorylated at the first position and at the 4' position on the non-reducing sugar [33]. Lipid A of *S. meliloti* is characterised by very long chain fatty acids [63]. The sugars that make up the core of the LPS of Rm1021 are glucose, galactose, mannose, Kdo and galacturonic acid [64]. Little is known about the composition of the O-antigen. *S. meliloti* can also produce K antigen. In Rm1021 this is a low molecular weight (3-6kDa) homopolymer of β-2,7 linked Kdo residues, believed to be attached to the membrane by a phospholipid anchor [65, 66]. In *S. meliloti* Rm1021 K antigen is produced at a lower level and has a lower molecular weight

than in some other strains where K antigen can act as a signal for the initiation of infection thread development [66]. In Rm1021 K antigen is symbiotically inactive.

As well as K antigen *S. meliloti* Rm1021 is able to produce two chemically distinct exopolysaccharides: succinoglycan (EPSI) and galactoglucan (EPSII). Succinoglycan has been the most extensively studied and has been found to be more efficient in mediating initiation of infection thread formation [67]. Succinoglycan is composed of octasaccharide repeat units consisting of one galactose and seven glucose units, with acetyl, succinyl and pyruvyl modifications (Fig. 1-14a and b) [68]. Under most conditions succinoglycan is synthesised in a high molecular weight (HMW) and a low molecular weight (LMW) form [69-71]. The LMW consists of monomers, dimers and trimers of the repeating unit with the trimer thought to be the active agent in initiating infection threads in alfalfa [69, 70]. Much like xanthan, succinoglycan is thought to adopt an ordered helical conformation, and this means the polymer behaves like a stiff rod [72, 73]. The ordered conformation and stiffness of the molecule is favoured in higher ionic strength solutions and by removal of charged constituents such as the succinyl groups [72, 73]. Galactoglucan is composed of a disaccharide repeat unit with an acetyl and pyruvyl modification [74] (Fig. 1-14c).

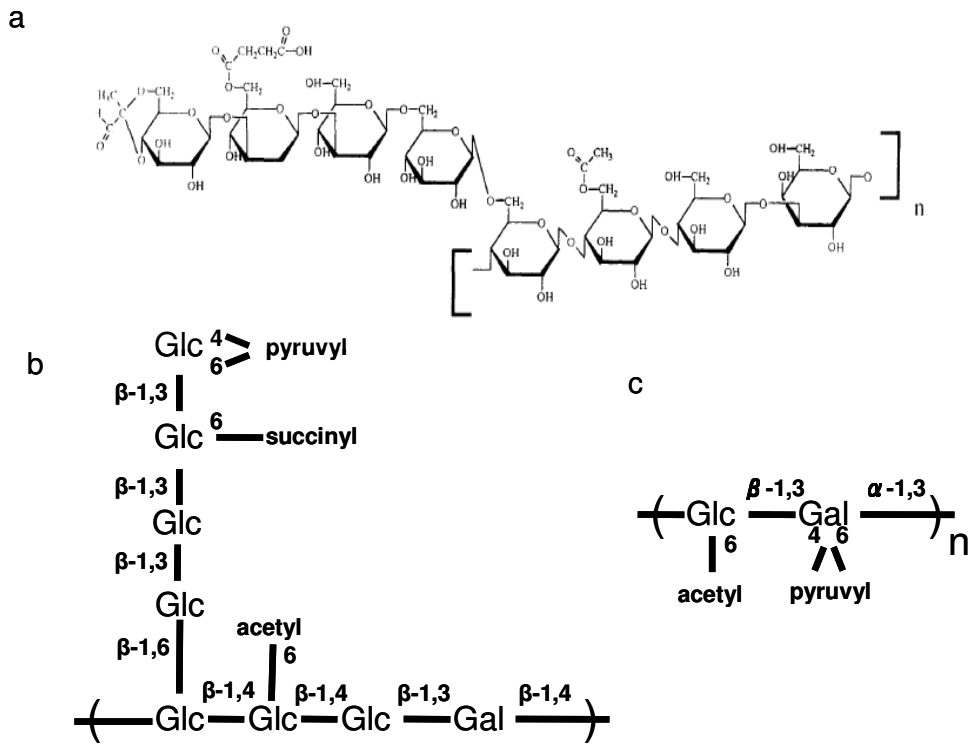


Figure 1-14. a, b. structure of succinoglycan. c. Structure of galactoglucan.

1.3.2 Succinoglycan biosynthesis

To understand the biosynthesis of succinoglycan a transposon mutant library was screened for succinoglycan production [59, 60]. Screening was made possible by the fact that succinoglycan fluoresces in the presence of the dye Calcofluor white, with succinoglycan mutants lacking fluorescence [60]. This screen allowed the identification of a cluster of succinoglycan biosynthetic genes (*exo*) located on the second megaplasmid of *S. meliloti*, pSymB [75]. In a similar mechanism to xanthan and other bacterial polysaccharides, succinoglycan is biosynthesised on undecaprenol lipid carriers in the cytoplasmic membrane of the cell [76]. Thanks to the structure of the repeating unit of succinoglycan being known and the identification of the *exo* genes, the biosynthetic pathway of succinoglycan was elucidated by analysing in vitro lipid

linked intermediates labelled with UDP-[^{14}C]galactose, formed by the *exo* mutants [77] (Fig. 1-15).

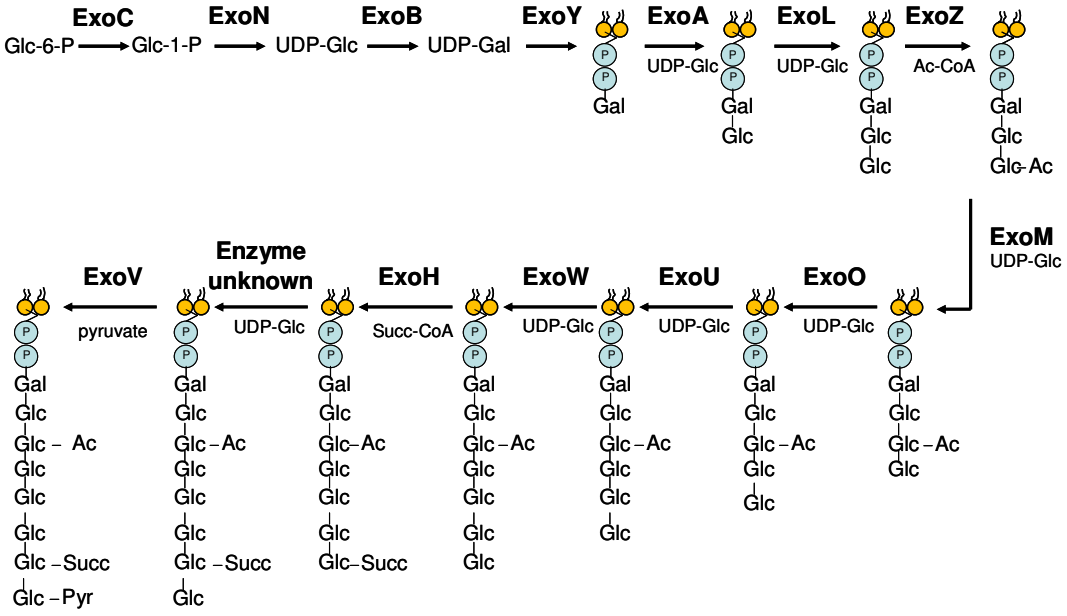


Figure 1-15. Biosynthetic pathway of the octasaachride repeating unit of succinoglycan. ExoC, ExoN and ExoB are responsible for synthesis of the activated UDP galactose. This is then loaded onto a phosphorylated isoprenoid lipid membrane carrier located on the inner face of the cytoplasmic membrane, where synthesis of the octasaccharide polymer occurs. After addition of pyruvate by ExoV the octasaccharide unit is flipped to the periplasm and subsequent polymerisation is carried out by ExoP, ExoQ and ExoT. Shown in diagram are phospholipids of inner membrane (yellow) and the phosphorylated lipid membrane carrier where the octasaccharide is assembled before flipping to the periplasm.

The precursors for succinoglycan biosynthesis, UDP-galactose and UDP-glucose, are synthesised by ExoC, ExoB and ExoN. ExoC is predicted to be a phosphoglucomutase which converts glucose-6-phosphate to glucose-1-phosphate [78]. The gene *exoN* encodes a predicted uridine diphosphoglucose pyrophosphorylase (UDPGP) which converts glucose-1-phopshate to UDP-glucose [79]. ExoB was found to be homologous to the *E. coli* GalE protein, a UDP-glucose-4-epimerase, which catalyses the conversion of UDP-glucose to UDP-galactose [80]. This activated form of galactose, UDP-galactose, is required to start the process of forming the octasaccharide repeat unit of succinoglycan for polymerisation [77]. The succinoglycan

biosynthetic pathway is shown in Figure 1-15. The initial step in the construction of the octasaccharide unit is carried out by ExoY, a galactosyl transferase that transfers galactose onto the lipid membrane carrier [77]. Acetylation, succinylation and pyruvylation of the chain are carried out by ExoZ, ExoH and ExoV respectively [77, 79, 81]. The complete octasaccharide unit is then flipped to the periplasm where polymerisation occurs. The polymerisation and biosynthetic control of the molecular weight of the final succinoglycan polymer is complex. This is due to the fact two species exist in the form of HMW and LMW succinoglycan and it is hypothesised that the two are produced by alternative mechanisms. Polymerisation of the octasaccharide unit is thought to occur via the action of ExoP, ExoQ and ExoT [82]. While an *exoP* mutant appeared to show no polymerisation at all, ExoQ appears to be essential specifically for formation of HMW succinoglycan while ExoT is needed to produce dimers and trimers of the repeating unit [82]. Control of the molecular weight of the mature polymer is further complicated by the fact that *S. meliloti* also makes two succinoglycan endo-1,3-1,4-β-glycanases ExoK and ExsH which break down HMW succinoglycan and contribute to the production of LMW succinoglycan [79, 83]. Interestingly both of the extracellular enzymes are only able to cleave succinoglycan effectively from *S. meliloti* cells that are actively synthesising the polysaccharide [84]. Both of the enzymes were inefficient when added to cell free preparations of succinoglycan [84]. It was also shown that the acetyl and succinyl modifications alter the action of the depolymerases, with the absence of the acetyl group enhancing susceptibility and the absence of the succinyl group decreasing susceptibility to depolymerisation [85].

1.3.3. Regulation of succinoglycan biosynthesis

As found in many bacteria, limitation of essential nutrients such as nitrogen, phosphorus and sulphur in a rich carbon source can massively increase exopolysaccharide production [53, 86]. Production of succinoglycan is modulated by the osmolarity of the medium, with larger amounts of HMW being produced by increasing the osmolarity [87]. Phosphate can also affect succinoglycan biosynthesis, with low (less than 0.05mM) and high (>150mM) phosphate concentrations increasing succinoglycan production while concentrations in between (the concentration of many laboratory media) reduce succinoglycan production [41].

At the metabolic level the *exoY* gene and its product protein are crucial points of succinoglycan biosynthesis control, as ExoY catalyses the transfer of UDP-galactose to the lipid carrier in the first step of succinoglycan production. The reaction catalysed by the ExoY protein has been shown to be regulated posttranscriptionally by interaction with the ExoX protein, which negatively controls succinoglycan biosynthesis [88, 89]. The *exoY* gene is also regulated at the level of transcription, with four possible promoters directing transcription of *exoY* [88, 90, 91].

The transcription of the succinoglycan biosynthesis genes is also altered by regulatory proteins ExoR and ExoS. The *exoR*95::Tn*5* and *exoS*96::Tn*5* mutants were both isolated due to the fact that they increased the fluorescence and mucoidy of the parent strain Rm1021 [86]. Subsequent analysis showed that the *exoS*96::Tn*5* mutant is a gain of function mutation, which results in upregulated transcription of succinoglycan biosynthesis genes and hence an increase in the level of succinoglycan produced [86, 92]. The *exoS* gene encodes for the sensor histidine kinase of a two component regulator system that acts in concert with a response regulator ChvI

effecting positive control on succinoglycan production by upregulating transcription of *exo* genes [92] (Fig. 1-16). It was shown that in the case of the *exoS*96::Tn*5* mutation upregulated succinoglycan biosynthesis was a result of the fact that the protein was still being synthesised but in a truncated form, which left it constitutively active [92]. As well as increasing succinoglycan production the *exoS* mutant also had reduced motility due to downregulation of flagella synthesis [93]. The *exoR*95::Tn*5* mutant was isolated at the same time as the *exoS*::Tn*5* mutant and has a similar phenotype, including upregulation of succinoglycan biosynthesis due to increased transcription of *exo* genes and downregulation of genes for flagella biosynthesis [86, 93]. Only recently it has been found that the ExoR protein, which is located in the periplasm, negatively regulates ExoS by binding to it in the periplasm [94, 95]. The environmental signal which may influence the activity of the ExoR/ExoS system is currently unknown [92, 94, 95].

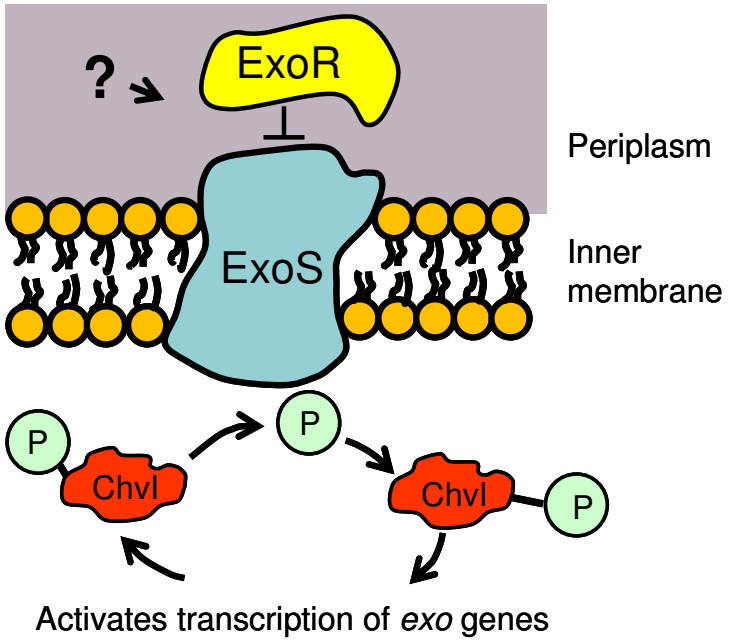


Figure 1-16. The ExoS two component regulator acts in concert with the response regulator to activate transcription of succinoglycan biosynthesis genes. ExoR negatively regulates the activity of ExoS. The environmental signal that controls the activity of this system is unknown.

1.3.4. Galactoglucan

As well as producing succinoglycan, *S. meliloti* is also capable of producing a second polysaccharide called galactoglucan or EPSII. Galactoglucan is also able to initiate infection thread formation in alfalfa and can act as a substitute in mutants that do not produce succinoglycan [96].

Under normal laboratory conditions *S. meliloti* Rm1021 does not produce galactoglucan. The ability of Rm1021 to do so was again discovered through the transposon mutant library screen where mutants were isolated as a result of a massive increase in mucoidy that did not fluoresce with calcofluor, suggesting production of a novel polysaccharide [96]. The production of EPSII turned out to be unrelated to the transposon; instead it was shown that a precise excision of an insertion sequence in the *expR* gene had restored the open reading frame of this gene [97]. ExpR was shown to be a LuxR homolog which activates transcription of genes in a density dependent fashion (although at much lower cell densities than most other LuxR systems), resulting in production of symbiotically active EPSII [97].

It has subsequently been discovered that Rm1021 with a non-functional *expR* gene can make EPSII at very low phosphate conditions [41]. However this EPSII is not symbiotically active, apparently due to the fact that it is HMW [41].

1.3.5. Aggregation in Rhizobia

As with *X. campestris*, *S. meliloti* aggregation is poorly studied. As well as the aggregate studies mentioned (section 1.1.2) whereby the related species *R. leguminosarum* is able to produce cellulose which increases aggregation, another strain of *R. leguminosarum* was studied for biofilm formation [98]. It was found that the bacteria formed a unique biofilm structure. Bacteria were seen to form aggregates

on the surface but all the cells attached to the surface by their poles and were arranged laterally compared to each giving the aggregates a 'honeycomb' looking structure (Fig 1-17) [98]. Analysis of exopolysaccharide mutants showed that exopolysaccharide production is essential for development of a mature biofilm [98]. Some studies have also been carried out on *S. meliloti* biofilms. It was found that the *nod* genes, which are essential for initiation of the symbiosis, are also needed for development of a mature biofilm [99]. During the course of this study it was also shown that the *exoS*95::Tn*5* and *exoR*96::Tn*5* mutants had enhanced attachment to a surface [95]. The *Rhizobium meliloti* strains studied by Deinema and Zevenhuizen (see section 1.1.2) did not appear to form aggregates in culture compared to the other strains [9].

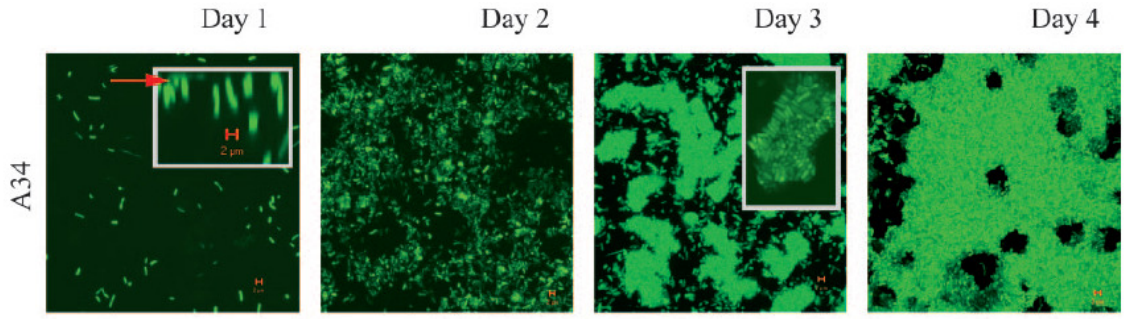


Figure 1-17. Formation of a biofilm by *R. leguminosarum*. GFP expressing cells sedimented onto a coverslip and were then imaged by confocal microscopy. Inserts show bacteria interacting with the surface via their poles and aligning laterally within the aggregates formed [98]. Scale bars=2µm.

The exopolysaccharides produced by *S. meliloti* Rm1021 are well studied and characterised. *S. meliloti* is thus an ideal system with which the role of exopolysaccharides in aggregation can be characterised at the structural and potentially molecular level.

1.4 Mechanisms underlying polysaccharide-induced bacterial aggregation

While observation of polysaccharides in aggregates gave strong support for the fact that polysaccharides were driving the aggregation of cells, the physical mechanisms underlying this process are also of interest. Understanding these mechanisms may aid in improving aggregation. For example in industrial processes such as activated sludge or in developing biotechnological technologies such as the use of bacteria for hydrogen production for fuel uses, aggregation of the bacteria is needed to improve fermentation of substrates or production of desired products [5, 100]. Such an understanding could also be of use in the successful production of bioflocculants to help reduce the need for environmentally harmful chemical flocculants currently used in industry [101]. An understanding of the polysaccharide-driven aggregation of bacteria could also be of use in reduction of aggregates and biofilms involved in disease or that are damaging to industrial processes.

1.4.1 Aggregation mechanism I: Polymer bridging

A hypothesis for the mechanism underlying polysaccharide-driven aggregation was first provided by researchers studying aggregation of activated sludge bacteria. The observation that production of extracellular polysaccharides appeared to result in the formation of a matrix around the cells, or that the aggregates were the result of adjoining capsules, gave rise to the idea that such aggregation was driven by a polymer bridging mechanism as shown in colloidal systems [5, 7, 102]. The idea of using colloidal systems to understand bacterial cultures is a common theme and dates back to the original studies on aggregation of bacteria in the late 1800's and early 1900's [6]. The polymer bridging mechanism was first shown to operate in

the aggregation of colloids by polymers by researchers interested in decontamination of wastewater by chemical means [103] (Fig 1-18).

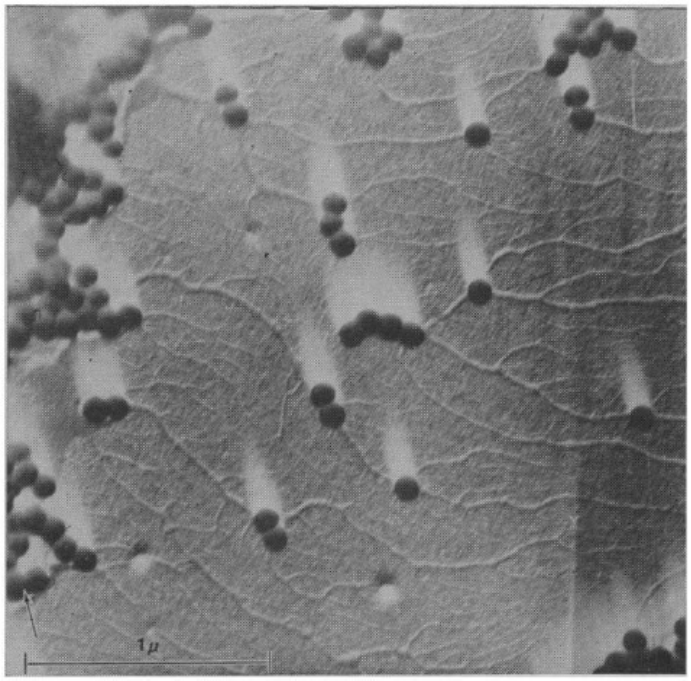


Figure 1-18. Formation of bridging aggregates by addition of a polymer to a colloidal system. Polymer fibrils are shown adsorbing to the surface of latex particles, which all appear to be associated with the fibrils rather than in the open spaces. Polymers can then draw the particles into aggregates [103]. Scale bar=1μm.

Polymer bridging results from the adsorption of polymer segments to the surface of the colloid or cell, with a portion of the polymer length extending into the solution which can then adsorb to another cell or particle, drawing them into an aggregate [5, 7, 102] (Fig. 1-20). The result of aggregation also means that the aggregates and cells will sediment out into a two phase system with a lower phase rich in cells and adsorbed polymer and an upper phase lacking these components. Both capsular polysaccharides and exopolysaccharides that adsorb to the surface of the cell with a high enough molecular weight would be able to mediate cell-cell interactions via this mechanism. This also served to explain the densely stained extracellular matrix or fibrils that surrounded various bacterial aggregates observed by electron microscopy.

Support for the hypothesis also came from the addition of chemical polymers and polysaccharides isolated from bacteria which resulted in the aggregation of bacteria and colloidal particles [5, 7, 8, 102]. One of the predictions of the hypothesis was that as the cell concentration increased the amount of polysaccharide needed to cause aggregation would also proportionally increase, which was shown to be the case by the addition of polymer to cultures [102]. It was also shown that an increase above the optimum concentration of polymer could also result in redispersal of the cells due to saturation of the cell surface with polymers preventing bridging from occurring [102].

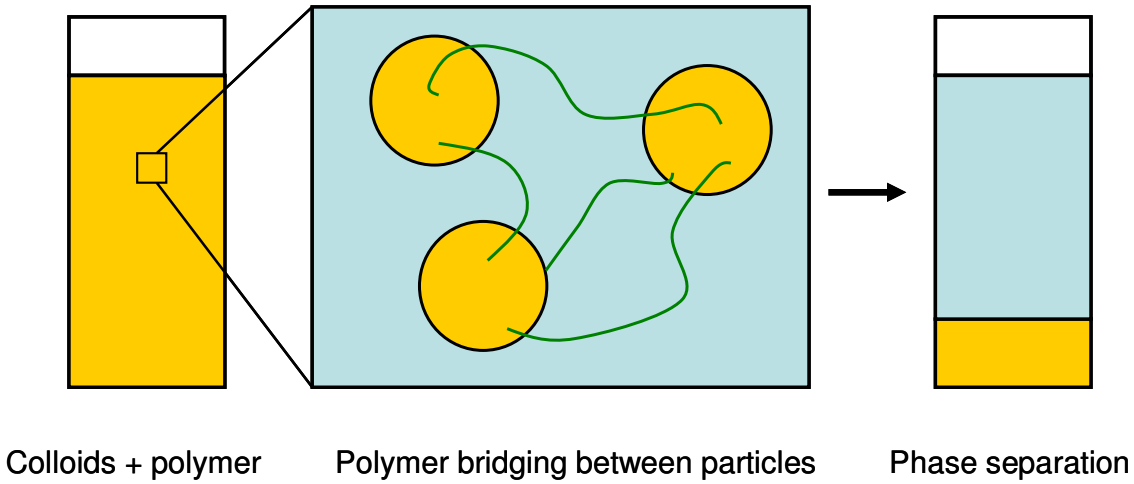


Figure 1-19. Diagram of polymer bridging. In a colloidal dispersion polymer can adsorb to the particles drawing them together into an aggregate. This then results in phase separation, with one of the phases rich in colloids and the bridging polymer.

1.4.1 Aggregation mechanism II: Depletion attraction

While polymer bridging appears to explain aggregation observed in many bacterial systems a second mechanism of polymer-driven colloid aggregation exists called depletion attraction, or as it is known in the biological literature, macromolecular crowding [104, 105]. Depletion attraction is based on the generation of

entropic forces driving aggregation of macromolecules or colloids due to crowding of the system by the addition of other constituents to the background solvent. These added constituents can be particles, rods or polymers, usually smaller than the macromolecules or colloids [106].

The depletion attraction phenomenon can be understood most easily by first considering a system composed of hard spheres mixed with a larger number of spheres of a smaller diameter (Fig. 1-20). The spheres are considered non-interacting or non-adsorbing and lack attractive interactions. As the spheres cannot interpenetrate each other; the closest the centre of mass of a small sphere can approach the surface of a larger sphere is equal to $\frac{1}{2}a_s$ (where a_s is the diameter of the small spheres and a_L is the diameter of the large spheres) (Fig. 1-20). This means that the large particles have an 'excluded volume' surrounding them, which is inaccessible to the centre of mass of the small particles. When the large particles approach each other, however, the excluded volumes overlap. This then increases the total volume available to the centre of mass of the smaller spheres, increasing their entropy (Fig. 1-20). This increase in entropy of the small spheres can drive an 'attractive' depletion force between the large spheres and maximise the entropy of the system as a whole (Fig. 1-20). Alternatively it can be said that interaction of the small spheres with the surface of the large reduces their entropy, so reducing that surface area by aggregation of the large spheres increases the entropy of the smaller spheres and maximises the entropy of the system. Interaction of the large particles with the surfaces of the container will also reduce the excluded volume of the particles surface and so depletion attraction will also drive interaction of the large spheres with the walls of the container [106].

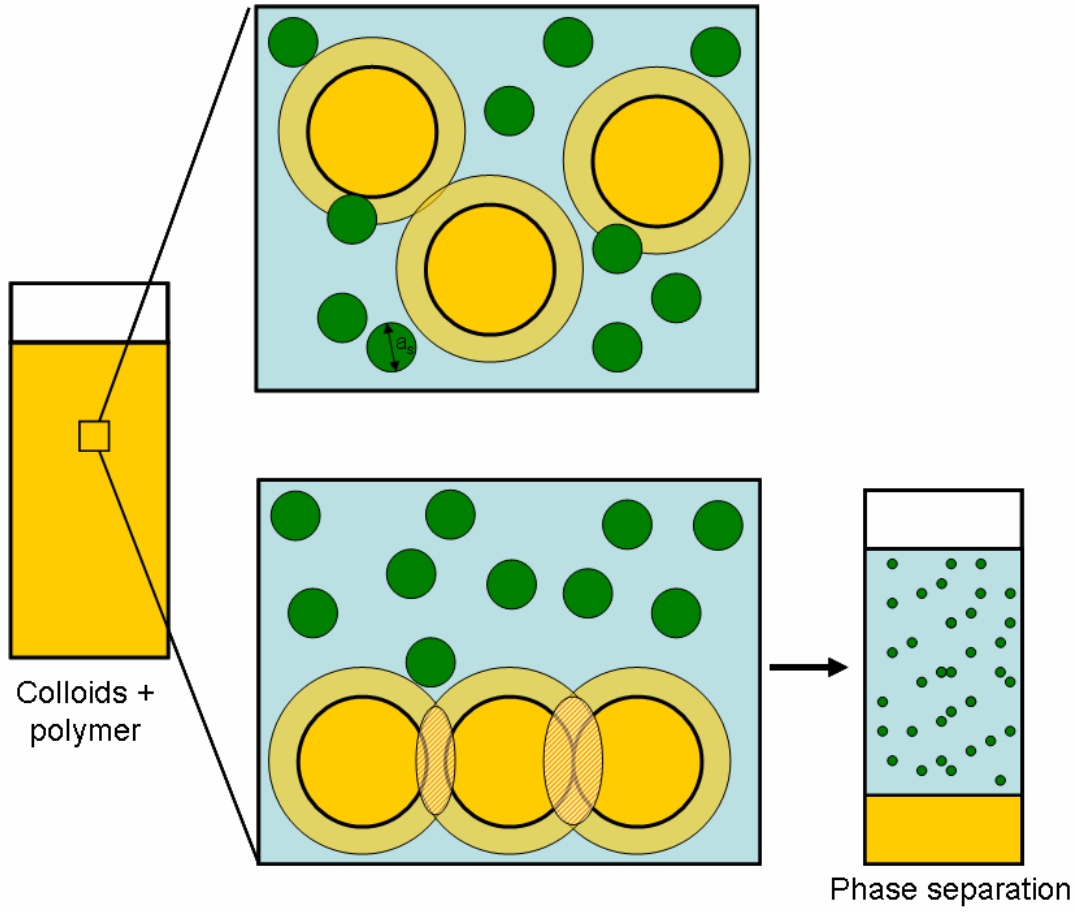


Figure 1-20. Depletion attraction. Shown are mixtures of large spheres (yellow) with non-adsorbing/non-interacting smaller spheres (green, diameter a_s). The closest that a centre of mass of the smaller spheres can get to the surface of a larger sphere is equal to 1/2 a_s. This then means that the yellow shaded regions are unavailable to the centre of mass of the smaller spheres, including an excluded volume around the large spheres. Aggregation of the large spheres creates an overlap of these excluded volumes (shown by the hashed regions in the lower panel). This then increases the volume available to the centre of mass of the smaller spheres increasing their entropy and maximising the entropy of the system as a whole. Aggregation of the particles can result in a phase separation. In this case however the phases will consist of a region rich in large spheres and a volume rich in the smaller spheres.

Depletion attraction is similar to the hydrophobic effect that can for example drive the organisation of proteins. While the hydrophobic effect is also driven by enthalpy effects of the hydrogen bonding of water, both mechanisms minimise the surface (exposed to a macromolecular solute or water) to maximise entropy [105].

Depletion attraction can also be understood in terms of the osmotic pressure of the system. When the large particles come closer together, if the gap between them is smaller than a_s, the smaller spheres are excluded from this gap. This creates an imbalance in osmotic pressure which results in the large particles being drawn together into an aggregate.

Much like polymer bridging, the formation of aggregates will lead to a phase separation. However in this case the separation will consist of one phase rich in colloids and the other phase rich in non-adsorbing polymer [107] (Fig.1-21).

1.4.2 Depletion attraction and biology

The addition of a crowding agent such as a polymer to a colloidal dispersion can give thus rise to aggregation of those colloids, even if the polymer does not adsorb to the surface of the particles. This interaction has been shown to be of relevance to biological systems albeit under the different label of macromolecular crowding. Macromolecular crowding has traditionally been used to model how the crowded environment of the cytoplasm of cells can alter reaction rates and equilibrium [108-110]. However it has also been modelled and shown experimentally to drive organisation of macromolecules within the crowded cytoplasm [105, 111-113]. For example, chloroplasts of algae and green plants contain membranes called thylakoids, which align laterally to form stacks called grana. Kim *et al.* isolated the membranes and were able to induce the same pattern of stacking by adding a crowding agent such as the polysaccharide dextran [111]. The stacking of the membranes was believed to maximise the volume available to the polysaccharide, increasing its entropy and maximising the entropy of the system as a whole [111].

Interestingly bacterial extracellular polysaccharides have also been shown to induce depletion attraction and aggregation in colloidal systems. As mentioned earlier (section 1.2.2) xanthan has important uses, for example in the food industry [55]. Xanthan is used as a thickener in salad dressings, which stabilises the emulsion by preventing the aggregation of emulsion droplets [114]. However it was found that by adding xanthan to the emulsion below this critical concentration actually resulted in an increase in the rate of creaming due to depletion attraction [114]. A similar effect was found by Cao *et al*., who used succinoglycan as well as xanthan and found similar results [115]. Extracellular polysaccharides produced by *Lactococcus* spp present in milk were able to induce depletion attraction and aggregation of colloidal components of milk, such as casein micelles [107, 116].

Given that depletion attraction will be a general phenomenon of crowded environments and that bacterial extracellular polysaccharides have been shown to induce depletion attraction it is perhaps surprising that it has not been studied in the context of bacterial aggregation. The production of extracellular polymers such as polysaccharides by bacterial cultures will lead to crowded dispersions and the bacteria will then be subject to the entropic forces generated by depletion attraction. For depletion attraction to occur the interaction between the polysaccharide and the cell surface would need to be incompatible, i.e. the polysaccharide would have to be non-adsorbing. In the case of secreted exopolysaccharides this does seem possible. Firstly in many cases the bacterial cell surface is polyanionic as are many extracellular polysaccharides (such as succinoglycan [68]). In physiological media however the salt concentration may be high enough to screen out the charges reducing electrostatic repulsion. However in this case interaction of the secreted

polysaccharide with the cell surface would still lead to an unfavourable loss of conformational entropy of the polymer. This could be overcome by the generation of favourable interactions with polymers on the cell surface. However, as mentioned earlier polysaccharides such as xanthan and succinoglycan tend to adopt ordered conformations [44, 45, 105]. To form similar interactions with the cell surface may therefore require the polymer to leave this conformation, which would also be energetically unfavourable. In the presence of exopolysaccharides, it seems likely that depletion attraction could be operating. The role this ubiquitous force may play in bacterial aggregation has not generally been addressed. Eboigbodin *et al*. studied the aggregation of *E. coli* (strain AB1157) by adding a polyanionic polymer polystyrene sodium sulfonate (PSS), which they stated showed evidence of depletion attraction [117]. Similarly Liu *et al*. looked at *E. coli* aggregation with the addition of DNA where depletion attraction was believed to play a minor role in the aggregation they observed [118].

1.5 Aims of this study

Thus far the role of depletion aggregation in bacterial cultures has been poorly studied. Depletion attraction occurs in any crowded dispersion and as such may also be important in a culture of bacteria producing exopolysaccharides. *S. meliloti* cultures will therefore be studied to assess whether production of the well-characterised exopolysaccharide succinoglycan can result in depletion attraction driving organisation of the cells. This system has the advantage that it is known that under most environmental conditions *S. meliloti* strain Rm1021 is only producing succinoglycan [71]. Succinoglycan is known to be secreted into the medium as an

exopolysaccharide. There are also a number of well characterised mutants that can alter both the level of production and characteristics of the molecule, which can be used to study the effects of succinoglycan in aggregation of *S. meliloti* Rm1021.

While *S. meliloti* had previously been shown not to aggregate during the exponential growth phase no studies appear to have been carried out regarding the ability of this species to form aggregates as the cells enter late exponential phase or stationary phase. The primary aims of this thesis were (i) to characterise whether *S. meliloti* Rm1021 was capable of aggregation in later growth stages and whether such aggregation could lead to the culture becoming unstable with a resulting loss in turbidity (ii) to characterise the role of extracellular polymers in this process, including the well-characterised succinoglycan, by utilising succinoglycan biosynthesis mutants.

During these screens it was found that an exopolysaccharide production mutant with a disruption in the *exoS* gene had enhanced aggregation. Further characterisation of the aggregation of this strain showed there were several features indicative of depletion attraction. Experiments will be described that test this hypothesis by looking at the concentration of cells and polymer required to cause phase separation. Investigation of how this depletion aggregation driven system of aggregation may affect the ability of *S. meliloti* to interact with *in vitro* surfaces and with those of its host will also be investigated and discussed.

Chapter 2

Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study and their sources are shown in Table 2-1, Table 2-2 and Table 2-3.

Table 2-1. *S. meliloti* strains used in this study

Strain	Description	Reference
Rm1021	Spontaneous SU47 mutant, Sm^R	61
Rm7210	Rm1021 *exoY*:: Tn*5* , Sm^R, Nm^R	77, 89
Rm7096	Rm1021 *exoS*::Tn*5*, Sm^R, Nm^R	86, 93
Rm8396	Rm1021 *exoS*::Tn*5-233*, Sm^R, Nm^R, Gm^R, Sp^R	86, 93
Rm7095	Rm1021 *exoR*::Tn*5*, Sm^R, Nm^R	86
Rm7094	Rm1021 *exoB*::Tn*5*, Sm^R, Nm^R	80
Rm8341	Rm1021 *exoZ*::Tn*5*, Sm^R, Nm^R	81
Rm7154	Rm1021 *exoH*::Tn*5*, Sm^R, Nm^R	119
Rm7445	Rm1021 *exoK*::Tn*5*, Sm^R, Nm^R	83
Rm7013	Rm1021 *exsH*::Tn*5*, Sm^R, Nm^R	83
Rm8826	Rm1021 *exoK*::Tn*5-233 exsH*::Tn*5*, Sm^R, Nm^R, Gm^R, Sp^R	83
Rm Δ*fliF*	Rm1021 Δ*fliF*, Sm^R	G. Ferguson
RmGD01	Rm1021 *exoS*::Tn*5-233*, *exoY*::Tn*5*, Sm^R, Nm^R, Gm^R, Sp^R	This study
RmGD02	Rm1021 *exoS*::Tn*5-233*, *exoZ*::Tn*5*, Sm^R, Nm^R, Gm^R, Sp^R	This study
RmGD03	Rm1021 *exoS*::Tn*5-233*, *exoH*::Tn*5*, Sm^R, Nm^R, Gm^R, Sp^R	This study

R denotes antibiotic resistance

Table 2-2. *E. coli* strains used in this study

Strain	Description	Reference
MG1655	$F^- \lambda^-$ *rph-1*	120
DH5α	F- φ80d*lacZ*ΔM15 Δ(lacZYA-argF)U169 *deoR recA1 endA*1 *hsdR*17($rk^- mk^+$) *phoA supE*44 λ^- thi^{-1} *gyrA96 relA*1	121
MM294A	*Pro*-82 *thi*-1 *end*A1 *hsdR*17 *supE*44	75
JW1922	BW25113 Δ*fliF,* Km^R	122
GD1922	MG1655 x P1(JW1922), Km^R	This study

Table 2-3. Plasmids used in this study

Plasmid	Description	Reference
pRK600	pRK2013 *npt* : : Tn*9*, Cm^R Helper plasmid	75
pHC60	Broad host range plasmid with GFP. A modified pSW213 plasmid, with the addition of the stability fragment from plasmid RK2 and the *gfp* gene Tc^R	123

R denotes antibiotic resistance

2.2. Culture of Bacteria

2.2.1 Culture media

Luria Bertani (LB) broth [124]: Difco bacto tryptone (10g), Difco Bacto yeast extract (5g) and NaCl (10g) dissolved in 1l dH_2O. The pH was adjusted to 7.5 with 5M NaOH and the media autoclaved (20 lb/in^2, 120°C, 15 minutes).

LB with magnesium and calcium (LB_{MC})[60]: LB broth, $MgSO4.7H_2O$ (2.5mM) and $CaCl_2.2H_2O$ (2.5mM). $MgSO4.7H_2O$ and $CaCl_2.2H_2O$ were prepared as 0.5M solutions and autoclaved (20 lb/in^2, 120°C, 15 minutes) before being added to LB.

Phosphate buffered saline (PBS): NaCl (8g), KCl (0.2g), Na_2HPO_4 (1.44g), NaH_2PO_4 (0.24g), dissolved in a final volume of 1l of dH_2O. pH adjusted to 7.5 with 5M NaOH and autoclaved (20 lb/in^2, 120°C, 15 minutes).

Phosphate Modified MOPS-MGS minimal medium [41]**:** 50 mM MOPS (morpholine propane sulfonic acid; adjusted to pH 7.4 and autoclaved), 55 mM mannitol, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 19 mM glutamic acid, and 0.004 mM biotin. Either 0 or 0.1 mM (low-phosphate) or 100 mM (high-phosphate) K_2HPO_4 was added.

M9 [77, 124]: 5× M9 salts (200ml), $MgSO_4$ (1mM), $CaCl_2$ (0.25mM), Biotin (1mg l^{-1}), mannitol (0.4% w/v) dissolved in a final volume of 1l of dH_2O.
5× M9 salts [124] were prepared by dissolving the following to a final volume of 1l: $Na_2HPO_4.7H_2O$ (64g), KH_2PO_4 (15g), NaCl (2.5g), NH_4Cl (5.0g). When making nitrogen-limited M9 the NH_4Cl was omitted.

Solid agars: LB_{MC}, LB and M9 media were converted to solid media by the addition of Difco agar (15g L^{-1}) prior to autoclaving.

2.2.2 Antibiotics

Antibiotics used in this study are shown in Table 2-4.

Table 2-4. Antibiotics used in this study

Antibiotic	Final concentration (μg ml^{-1})		Solvent
	S. meliloti	*E. coli*	
Kanamycin		50	Water
Streptomycin	500		Water
Neomycin	200		Water
Spectinomycin	100		Water
Gentamycin	50		Water
Chloramphenicol		12.5	Ethanol
Tetracycline	10	10	50% Ethanol

2.2.3 Growth of bacterial cultures

S. meliloti: Cultures were grown with shaking on an orbital shaker (200rpm) at 30°C. Initially, 5ml cultures were grown in test tubes in LB_{MC} for 24 hours (with the addition of antibiotic relevant for that strain), after inoculating a single colony from a LB_{MC} agar plate. This culture was then sub-inoculated into fresh LB_{MC} at an OD_{600} 0.1, followed by incubation to the required growth phase (late exponential phase ~15 hours). For large-scale cultures LB_{MC} (>100ml) 5ml of culture of that strain was grown from single colony (with antibiotics) for 24 hours. This was then sub-inoculated into 100ml fresh LB_{MC} followed by incubation for ~9 hours to exponential

phase. This culture was then sub-inoculated into the required amount of fresh LB_{MC} at an OD_{600} 0.1 and grown to the required growth phase.

For growth in minimal media the initial 5ml culture was first washed by centrifugation and the pellet resuspended in the medium that would be used for subsequent incubation. This suspension was then inoculated into fresh media at an OD_{600} 0.1 (M9 and phosphate modified MOPS) or an OD_{600} 0.5 (nitrogen limited M9), followed by incubation to the required growth phase.

E. coli. Cultures were grown with shaking on an orbital shaker (200rpm) at 37°C. Initially 5ml cultures were grown in the presence of the relevant antibiotic from a single colony isolated from a LB agar plate overnight. The culture was then sub-inoculated into fresh LB at OD_{600} 0.1 and grown to the required growth phase.

When measuring growth of the bacterial strains, the bacteria were grown as indicated above, with OD_{600} being monitored over time. Colony forming units were assessed by removing 200µl of culture at each time point, serially diluting and then transferring 3 10µl spots for each dilution onto LB_{MC} (*S. meliloti*) or LB (*E. coli*) agar plates.

2.2.4 Storage of bacterial cultures

For long term storage of *S. meliloti*, 900µl of fresh culture of bacteria, grown for 2 overnights in LB_{MC} supplemented with antibiotic was mixed with 100µl of DMSO and stored in a sterile vial at -80°C. Cultures were recovered by transferring the bacteria from the vial using a sterile loop and streaking the bacteria onto LB_{MC} agar plates with appropriate antibiotic. Plates were incubated for 4 overnights at 30°C wrapped in aluminium foil. Single colonies could then be picked for growth in liquid culture. *S. meliloti* were stored on the plates for ~4 weeks at 4°C.

E. coli was stored in a similar way, except that recovery for short term storage on plates was on LB agar plates at 37°C for 1 overnight.

2.3 Construction of double mutants and strains expressing GFP

2.3.1 Transduction with bacteriophage M12 and S. meliloti

Preparation of lysates of bacteriophage M12: Fresh cultures of the required donor strain of *S. meliloti* were grown for 2 overnights in LB_{MC}. This culture was then diluted 1:10 into two tubes of 5ml LB_{MC} (the second culture served as a negative control) and incubated at 30°C for 2 hours. To one of these tubes 100µl of M12 bacteriophage Rm1021 lysate was added. Both the control culture and the culture with the applied phage were then incubated overnight at 30°C. Following incubation successful lysis was evident as a clear sample, with the lysed bacteria forming a white precipitate compared to the cloudy negative control. Chloroform (500µl) was added to the sample, which was then vortexed and left to settle for 30 minutes. The sample was then centrifuged at 7000×g for 10 minutes. The top layer of the sample was then carefully removed with care being taken to ensure that the dead bacteria at the bottom of the tube were not disturbed and transferred to a sterile tube. Chloroform was added to the removed lysate (10% of the total volume removed), which was then stored at 4°C.

Transduction with bacteriophage M12 lysates: The recipient strain that the mutation was to be traduced into was grown for 2 overnights in LB_{MC}. The recipient strain (100µl) was then mixed with 100µl phage in an eppendorf, followed by 3 serial dilutions to give 10^{-1}, 10^{-2} and 10^{-3} dilutions of cells and phage. Controls of lysate and cells alone were also placed into two additional eppendorfs. Samples were

vortexed and incubated at 30°C for 2 hours, followed by incubation at room temperature for 30 minutes. Cells were then pelleted by centrifugation at 13000×g for 2 minutes in a bench-top centrifuge. The supernatant was removed and discarded and the cells were resuspended in 1ml of LB. The cells were pelleted by centrifugation at 13000×g for 2 minutes and resuspended in 200µl of LB. The cells were then recovered by incubation at 30°C with shaking at 200rpm for 1.5 hours. Cells were then plated on LB agar plates with the appropriate antibiotic and incubated at 30°C for 72 hours. Any colonies were further purified initially on a LB agar plate with the appropriate antibiotic, followed by growth on a LB_{MC} agar plate with the appropriate antibiotic selection.

2.3.2 Transduction with bacteriophage P1 and E. coli

Phage buffer: Na_2HPO_4 anhydrous (7g), KH_2PO_4 anhydrous (3g), NaCl (5g), 0.1M $MgSO_4$ (10ml), 0.1M $CaCl_2$ (10ml), 1% (w/v) gelatine (10ml), dissolved in dH_2O to a final volume of 1 litre.

Top agar: contained 0.3% (w/v) difco agar added to LB.

Preparation of lysates of bacteriophage M12: Fresh cultures of the required donor strain of *E. coli* were grown for 1 overnight in LB. This culture was then diluted 1:100 (volume) in 10mls of LB and grown to an OD_{600} of 0.8. Once the cells had reached the required OD600, 1ml was centrifuged at 13000×g for 1 minute, the supernatant was discarded and the cells resuspended in 1ml of fresh LB. Six eppendorfs were prepared containing $CaCl_2$ (50mM) and $MgSO_4$ (100mM) made up to a total volume of 200µl with sterile dH2O, five of which had 100µl of the cells and 5µl of P1 phage lysate added. One eppendorf was set up containing phage only as a control. These mixtures were then incubated 37°C for 25 minutes, before being

added to molten top agar, which were poured on LB agar plates containing $CaCl_2$ (0.5mM). Plates were incubated overnight at 37°C. Successful lysis was evident as plaques in the bacterial lawn (absent in the control). Phage buffer (2.5ml) was added to each plate. The top agar was then scraped from the plate using sterile slides and put into a sterile falcon tube. Chloroform (total 2.5ml=0.5ml per plate) was added to the falcon tube which was then vortexed and left to settle for 30 minutes. The sample was then centrifuged at 3000×g for 10-15 minutes, or until the supernatant was clear of agar. The supernatant was transferred to a fresh sterile tube along with 1ml of chloroform. The centrifugation was repeated, and the supernatant transferred to a fresh sterile tube, followed by storage at 4°C.

Transduction with bacteriophage P1 lysates: The recipient strain was grown overnight at 37°C and used to inoculate 10ml LB (1:100 dilution) in 1 100ml flask. The culture was grown to an OD_{600} of 0.4 and 1ml was centrifuged at 16 100×g for 1 minute. The pellet was resuspended in 1ml LB and 100µl of cells were transferred into each of 3 tubes containing $CaCl_2$ (50mM) and $MgSO_4$ (100mM) made up to a final volume of 200µl. To these 3 tubes either 10µl, 1µl or 0.1µl, of the appropriate P1 lysate was added and mixed before incubation at 37°C. After 20 minutes 200µl 1M sodium citrate and 500µl LB was added, followed by a further incubation for 30 minutes at 37°C. The cells were then centrifuged as before and the pellet resuspended in 100µl LB, plated on LB agar with the appropriate antibiotic selection, which was incubated at 37°C until colonies appeared, which were then purified and streaked onto LB agar plates.

2.3.3 Conjugation of pHC60 plasmid from E. coli *into* S. meliloti *using triparental mating*

Fresh 5ml *S. meliloti* cultures were grown for 2 overnights in LB_{MC}. The donor strain *E. coli* DH5α and helper strain *E. coli* DH5α carrying the pRK600 plasmid were inoculated the day after the *S. meliloti* cultures and grown for 1 overnight in LB. After incubation 1ml of the cultures were centrifuged at 13 000×g for 1 minute and the pellet resuspended in fresh LB. This centrifugation step was repeated. The recipient S. meliloti culture (40µl), the donor strain *E. coli* DH5α carrying the pHC60 plasmid (40µl) and the helper *E. coli* DH5α carrying the pRK600 plasmid (40µl) were then mixed in an eppendorf tube by vortexing. This 120µl mixture was transferred onto a LB_{MC} plate and incubated overnight at 30°C. After incubation cells were transferred onto selective LB_{MC} agar plates containing streptomycin at twice the normal concentration and tetracycline in order to purify *S. meliloti* Rm1021 cells containing the pHC60 plasmid. Plates were incubated at 30°C for 4 overnights. Two further purifications were carried before growth on LB_{MC} plates containing streptomycin and tetracycline. Single colonies were isolated and transferred to 5ml LB_{MC} containing streptomycin and tetracycline, grown for 2 overnights, checked for GFP fluorescence by microscopy and transferred to sterile vials with DMSO for storage at -80°C.

2.4 Microscopy

2.4.1 Phase contrast and fluorescence microscopy

Cultures of the strains required for observation were grown to the required growth phase in media and conditions indicated and 5µl was spotted onto glass slides, with a

coverslip. Coverslips were sealed with clear nail varnish. Phase contrast microscopy images were taken with a 100x oil immersion phase contact lens. Fluorescence images were taken with a 100x oil immersion phase contact lens using UV excitation and DAPI filters. All phase contrast and fluorescence microscopy was carried out on a Carl Zeiss Axioskop microscope and Axiocam using Metamorph software.

2.4.2 Confocal microscopy

Confocal microscopy was carried out on an inverted confocal scanning laser microscope (Nikon TE300) using a 60x water immersions lens. Images were acquired by scanning the cultures with optimal settings for GFP (488nm excitation argon laser line and 505nm long-pass emission) and using Lasersharp 2000 software. Profiles of the xz plane and 3D images were obtained by sequential scans of the xy plane along the z axis, each image separated by 0.5μm. Images were subsequently processed using ImageJ 1.40g software [125]. 3D images were constructed using the volume viewer V1.31 plugin for the ImageJ 1.40g software [126].

Cells were incubated in chambered cover glass slides containing a borosilicate glass base 1μm thick (Lab-Tek Nunc; no. 155411) [98]. Confocal images were acquired form bacteria carrying the pHC60 plasmid which results in constitutive expression of GFP [123]. GFP-labelled cultures were grown to late exponential phase and transferred into the chambered coverslides and incubated without shaking at 30°C for the time indicated before shaking. In order to reduce evaporation from the chambers, incubation was carried out in humid Petri dishes. Where indicated the cultures were diluted prior to being transferred into the chambers.

2.5. Characterisation of motility and cell size

2.5.1 Imaging presence of flagella by transmission electron microscopy (TEM)

To assess motility of the different bacterial strains, cultures were grown to late exponential phase in the appropriate growth medium. Soft agar motility plates, consisting of 0.3% (w/v) agar added to LB prior to autoclaving (with the addition of $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$ to a final concentration of 2.5mM post autoclaving when assessing *S. meliloti* motility) were then inoculated with 5µl of culture of the chosen strain. Plates were then incubated at 30°C (*S. meliloti*) or 37°C (*E. coli*) and motility was assessed after the time indicated.

For visualisation of flagella on *S. meliloti* Rm1021 and the Rm1021 Δ*fliF* mutant by TEM, bacteria were grown to exponential phase in LB_{MC}. Cultures were then washed by centrifuging at 3000×g for 3 minutes and the pelleted cells were resuspended in sterile dH_2O twice. Samples were then transferred to carbon-coated copper grids and negatively stained for 2 minutes with uranyl acetate. Images were acquired using a Phillips CM-12 TEM operated at 200kV.

2.5.2 Measuring cell size

Cultures were grown in LB_{MC} to late exponential phase. Samples were then transferred directly to carbon-coated copper grids and negatively stained for 2 minutes with uranyl acetate for imaging by TEM. Images were analysed and measured using Openlab 2.2.5 software. Lengths and widths of cells were measured by clicking measuring software on images. At least 100 cells were measured per strain.

2.5.3 Electrophoretic mobility

Electrophoretic mobility was measured using a phase-amplitude light scattering (PALS) zeta potential analyzer (Brookhaven Zeta PALS, U.K.) based on the method used by Eboigoidin et al. [117]. Cultures of the required strains were grown in LB_{MC} to late exponential phase. The cultures were then centrifuged at 13000×g for 2 minutes. The pellet was resuspended in PBS. This washing step was repeated twice. Measurements were conducted using an electric field of 4 V cm^{-1} at a frequency of 2.0 Hz.

2.6 Aggregation measurements

2.6.1 Sedimentation screen

The different strains were assessed for aggregation by monitoring of sedimentation. The bacterial strains were grown to the required growth phase in the medium indicated. 1ml of the culture was then transferred to a cuvette which was covered with parafilm and incubated for 24 hours at 30°C without shaking. Images were acquired using a digital camera (Nikon). Aggregation of the strains was also assessed by phase contrast microscopy (section 2.4.1, page 51).

To assess the rate of sedimentation of the *S. meliloti* strains, three cultures of each strain were grown to late exponential phase and transferred to a cuvette which was covered with parafilm. OD_{600} was then monitored over the period of incubation at the time points indicated.

The above screen was also carried out with the *S. meliloti* strains dispersed in various preparations of succinoglycan. Supernatant from the *exoS* mutant, which contains succinoglycan was obtained from a 10ml late exponential phase culture and

centrifuged at 8000×g for 5 minutes. The resulting supernatant was removed and filter sterilised through a 0.2µm pore filter. Strains of *S. meliloti* to be tested in the presence of *exoS* supernatant were grown to late exponential phase, 1ml was then centrifuged at 13000×g for 2 minutes and the pelleted cells were dispersed in the *exoS* supernatant. Each strain was also centrifuged at 13000×g and then resuspended as a control. The sedimentation screen was then carried out as detailed above. Aggregation was also assessed by phase contrast microscopy.

The aggregation of the strains was also tested in the presence of cetrimide (hexadecyltrimethylammonium bromide)-precipitated succinoglycan. A 10ml culture of the *exoS* mutant grown to late exponential phase was centrifuged at 8000×g for 5 minutes. The resulting supernatant was removed and 0.3 volume of 1% (w/v) cetrimide was added. This was centrifuged at 8000×g for 5 minutes. The supernatant was removed and filter sterilised through a 0.2µm pore filter. The remaining pellet was resuspended in fresh LB_{MC} and filter sterilised through a 0.2µm pore filter. Strains of *S. meliloti* grown to late exponential phase in LB_{MC} were then centrifuged and the pellet resuspended in either the supernatant or the resuspended cetrimide pellet from the *exoS* mutant. The sedimentation screen was then carried out as detailed above.

The aggregation of the strains was also tested in the presence of *exoS* supernatant after the cells had been heat treated. Late exponential phase cultures of the parent strain Rm1021 and the *exoS* mutant were heat treated by incubating the cultures at 60°C for 1 hour. Cell viability was assessed by counting CFU's before and after heat treatment. The sedimentation screen was then carried out as detailed above. Additionally the parent strain Rm1021 was also tested in the presence of *exoS*

supernatant, isolated as detailed above. The sedimentation of the parent strain Rm1021 was also assessed after centrifuging the culture at 13000×g and then resuspending the cells in their own supernatant, as a control. Aggregation was also assessed by phase contrast microscopy.

2.6.2 Phase diagrams

Phase diagrams were used to assess polymer concentration dependence of destabilisation of the cultures [117]. The *S. meliloti exoY* mutant was grown to late exponential phase or *E. coli* MG1655 and MG1655 *fliF* mutant were grown to late exponential or stationary phase. The cultures were then centrifuged at 13000×g for 30 minutes in pre-weighed sterile centrifuge flasks. The supernatant was removed and the flask weighed to give the weight of the pellet. PBS or sterile dH2O was then added to give a final concentration of 25% (w/v) of cells. In cuvettes cells, polymer (succinoglycan, xanthan and PSS) and either PBS or dH2O were added in required volumes to give final concentrations of 2.5% to 20% (w/v) of cells and 0.05% (succinoglycan and xanthan) or 0.5% (PSS) (w/v) of polymer in a total volume of 1ml per cuvette. For each concentration of cells used a control with no added polymer was also made. The cuvettes were then sealed with parafilm and incubated without shaking for 24 hours at 30°C. Phase separation was assessed by either visual observation of the cuvettes or where indicated measurement of the OD_{600} of the cleared upper phase, after 24 hours of static incubation. To find the limiting polymer concentration the cuvettes were diluted by first mixing the contents of the cuvette, then removing 100µl and adding 100µl of fresh dH2O or PBS. After each dilution the cuvettes were incubated without shaking for 24 hours before visual observation

of the phase separation or where indicated, measurement of the OD_{600} of the upper phase.

2.7 Succinoglycan isolation, quantification and molecular weight

2.7.1 Quantification of succinoglycan production by the anthrone-sulphuric acid assay

Succinoglycan was purified from culture supernatants by precipitation by cetrimide (hexadecyltrimethylammonium bromide) and quantified using the anthrone-sulphuric acid assay [86]. The basis of the assay lies in the ability of a carbohydrate to form a furfural derivative in the presence of acid, which reacts with anthrone to form a blue-green colour which can be measured on a spectrophotometer [86]. This is used to quantify the polysaccharide by comparison with a standard curve prepared with known concentrations of glucose. Three 10ml *S. meliloti* cultures for each strain were grown to the required growth phase in the medium indicated. The cultures were then centrifuged for 1 hour at 13000×g. The supernatant was removed and 0.3 volumes of 1% (w/v) cetrimide was added to precipitate the succinoglycan. The precipitate was pelleted by centrifugation at 13000×g for 1 hour and redissolved in 1ml of 10% (w/v) NaCl.

This was then diluted 1:10 (volume) in a total volume of 2ml in a test tube and assayed by the anthrone-sulphuric acid assay. Acetone (0.5ml) was carefully layered on the top of the sample. Sulphuric acid (5ml) was then added to the test tube which was mixed slowly until a floc of anthrone appeared. The samples were then vortexted briefly and placed on ice for 5 minutes. Absorbance was measured at 620nm. A

standard curve was generated from glucose ranging from 0-40μg ml^{-1}. Succinoglycan production was normalised to OD_{600} of the culture.

2.7.2 Isolation and purification of succinoglycan

A 500ml culture of the *exoS* mutant was grown to late exponential phase in M9. This was centrifuged at 13000×g for 1 hour. For the phase separation experiments (section 2.6.2) the supernatant was then treated by ultrafiltration with ultrafiltration filter discs with a MWCO of 300kDa (Millipore). Overnight Proteinase K treatment at 37°C was followed by ultradialysis with at least 1l of dH_2O. The resulting solution was then lyophilised and stored at -20°C. Solutions were made in PBS or dH_2O to the required concentration from the dried material. Contamination from DNA or proteins also present in the succinoglycan sample was measured using a nanodrop spectrophotometer (Nanodrop 1000, Thermoscientific).

An alternative method of succinoglycan isolation was also attempted. The supernatant from the *exoS* mutant M9 culture was first lyophilised and the resulting dried material then resolubilised in 1/10 of the original volume. This was then treated with proteinase K at 37°C overnight followed by exhaustive dialysis (SpectraPor dialysis membrane, MWCO 1000) against dH_2O for 4 days. This was then lyophilised and stored at -20°C. Solutions were made in PBS or dH_2O to the required concentration from the dried material.

2.7.2 Measurement of molecular weight of succinoglycan

Gel filtration chromatography: Sample for the chromatography was prepared from the supernatant of the *exoS* mutant. A late exponential phase culture was grown in LB_{MC}. Cells were pelleted by centrifugation at 13000×g for 30 minutes and the

resulting supernatant then underwent dialysis (SpectraPor dialysis membrane, MWCO 1000) for 4 days against dH_2O. This was then lyophilised and the dried material was resuspended in 1/10 of the original volume of PBS. The sample was applied to a Sepharose 2B (Sigma-Aldrich) gel filtration column (Pharmacia Biotech XK26) at a rate of 0.2ml min^{-1} by a peristaltic pump (Gilson). Fifty 5ml fractions were collected by a fraction collector (FRAC-100, Pharmacia), which were then assayed by the anthrone-sulphuric acid assay at an absorbance of 620nm. The void and inclusive volume of the column were determined by blue dextran (Sigma) and glucose respectively.

Light scattering: Light scattering measurements were carried out on succinoglycan samples isolated from M9 supernatant and dissolved in PBS as described above. Light scattering measurements were carried out at 25°C with a helium-neon laser (λ= 632.8nm) at four different polymer concentrations (0.2, 0.3, 0.4 and 0.5 g l^{-1}) using an ALV-CGS3 unit from ALV-Laser Vertiebsgesellschaft m.b.H.

2.8 Assay of nodulation of alfalfa plants by *S. meliloti*

Jensens agar: $CaHPO_4$ (1g), $FeCl_3.6H_2O$ (0.1g), NaOH (100µl 10N NaOH) 1ml trace minerals, $MgSO_4$ (0.8mM), K_2HPO_4 (1.1mM), NaCl (3.4mM) dissolved in a total volume of 1l of dH_2O. For agar plates 15g of Bacto agar was added prior to autoclaving.

Trace minerals: H_3BO_3 (0.5g), $ZnSO_4.7H_2O$ (0.5g), $CuSO_4.5H_2O$ (0.25g), $MnCl_2.4H_2O$ (0.25g), $NaMoO_4.2H_2O$ (0.5g) dissolved in a total volume of 500ml.

Germination of seedlings: The surface of the alfalfa seeds were sterilised by washing in 5ml of sterile dH2O and 5ml bleach for 15 minutes, followed by exhaustive washing with sterile dH2O. The required number of seeds were spread

out on 1% (w/v) agar plates, which were then wrapped in tin foil, stood upright and incubated at 25°C for three days. After germination the seedlings had their seed coats removed by carefully using tweezers to remove them from the seedling. The seedlings were then placed on plates poured with Jensen's agar (40ml per plate), with the seedlings head sticking out of the plate via a small notch removed from the plate to allow plant growth out of the Petri dish. Late exponential phase bacterial cultures of the required strains were diluted to an OD_{600} 0.05 and 1ml was then transferred onto the roots of the seedlings. Plates were wrapped in parafilm and covered with parafilm followed by incubation at 25°C in a plant incubator (12 hours light) for 4 weeks. After 4 weeks of incubation the plants were measured for growth, condition of the plant, number of nodules and colour of those nodules. Photos were taken of the plants and of the nodules present on the roots (Nikon).

2.9 Assays for attachment to surfaces

2.9.1 Crystal violet microtitre plate assay

This assay was adapted from a previously described method [30]. Bacteria were grown to late exponential phase in LB_{MC} or nitrogen limited M9 and transferred to 96-well polystyrene round-bottom microtitre plates (Greiner), covered with a breathable seal. Each well was inoculated with 200 µl of stationary phase culture diluted to OD_{600} 0.02, with the appropriate antibiotic selection (Table 2.1). The microtitre plates were incubated at 30 °C for 24-72 hours without shaking. Plates were either incubated in humid Petri dishes with wet paper towels to reduce water loss or in Petri dishes without paper towels to enhance evaporation. Growth of the cultures was determined by measuring the OD_{595} of all wells with a microtitre plate reader (Bio-Tek,

Winooski, USA, model Elx800) prior to visualisation by crystal violet staining. After incubation, media was poured from the wells and the plates were then left to dry, by being inverted on a paper towel for 5 minutes. Biofilms were stained with a crystal violet solution (1% w/v); 220 µl was added to each well and left to incubate at 30°C for 30 minutes. The wells were then rinsed thoroughly and left to dry on paper towels. Stained biofilms were imaged by photography (Coolpix 4500, Nikon). The biomass of the attached cells was estimated by solubilising the crystal violet stain with 220µl of 33% acetic acid (v/v) and measuring the absorbance of the solubilised stain at 595nm on a plate reader.

2.9.2 Assay for attachment of cells to roots

An assay for attachment to plant roots was modified from Walker et al. [127]. Seedlings were germinated over 3 days and placed on Jensens agar plates and inoculated with the bacterial strains as described above. Plants were incubated for 3 days at 25°C in a plant incubator (12 hours light). Roots were then excised from the plant and weighed. This was followed by washing of the roots in sterile dH_2O to remove non-attached cells. The roots were then homogenised in 1ml of PBS with a tissue grinder. This suspension was serially diluted and plated on LB_{MC} agar to determine cell counts which were then normalised to the fresh weight of the excised roots.

Chapter 3

The *exoS* mutant has enhanced aggregation

In this study the role of succinoglycan in the aggregation of *S. meliloti* is being investigated. *S. meliloti* is known to synthesise two distinct exopolysaccharides, succinoglycan and galactoglucan (EPSII) [96]. Succinoglycan is the better characterised of the two polysaccharides produced by *S. meliloti* Rm1021. This is related to the fact that in Rm1021 EPSII is only produced in specific strains (designated Rm1021 *expR*+) [97] or under specific environmental conditions (media with less than 0.1mM or no phosphate present) [41]. This means that under most laboratory conditions EPSII is not being produced by Rm1021. Succinoglycan has also been more intensively studied because it appears to have a dominant role in signalling during initiation of the symbiosis with the plant host [67] and may also be of use in industrial applications [73]. As a result a number of studies have led to the development and characterisation of succinoglycan biosynthesis mutants that are altered not just in the levels of succinoglycan production but also in the structure of the molecule synthesised [58, 77, 79, 81-83, 85, 86, 92, 119, 128-131].

The initial aims of this study were to characterise whether succinoglycan actually induces aggregation in *S. meliloti*. Further to this it was hypothesised that if succinoglycan was causing aggregation that mutants altered in specific structural modifications of the polysaccharide might perturb aggregation. Surprisingly, despite the wealth of knowledge about the biosynthesis and structure of succinoglycan produced by *S. meliloti* Rm1021, before this study there were no reports regarding aggregation or biofilm formation by succinoglycan in *S. meliloti*. Here it is shown

that succinoglycan can induce aggregation in *S. meliloti* Rm1021, but only in mutant strains that overproduce succinoglycan.

3.1 Screening of exopolysaccharide mutants

Strains that were used in the screen are shown in Table 3-1. After growth of the different exopolysaccharide mutants of *S. meliloti* it was noted that the strains appeared to sediment at different rates. To investigate this further, the exopolysaccharide mutants were screened for differences in sedimentation rate. The cultures were grown to late exponential phase, transferred into cuvettes and left without shaking for 24 hours. As the cells start to sediment due to gravity, a clear upper phase is left at the top of the cuvette. Two succinoglycan mutants (mutants with transposon insertions in the *exoS* or *exoR* gene) were found to have an increased rate of sedimentation, with the result that after 24 hours all of the cells have settled to the bottom of the cuvette leaving an almost completely clear upper phase (Fig 3-1). In contrast the parent strain Rm1021 and the other mutants have a far slower rate of sedimentation (Fig. 3-1). After 24 hours this results in some clearance of the upper phase, but the bulk of the cells have remained in the culture as a stable, turbid dispersion (Fig. 3-1).

Table 3-1. Strains used in aggregation screen with description of genotype and phenotype.

Strain	Genotype	Phenotype	Reference
Rm1021	Rm1021	Parent	61
Rm7210	Rm1021 *exoY*:: Tn*5*	Succinoglycan biosynthesis abolished	77, 89
Rm7096	Rm1021 *exoS*::Tn*5*	Overproduction of succinoglycan Loss of motility	86, 93
Rm8396	Rm1021 *exoS*::Tn*5-233*	Overproduction of succinoglycan Loss of motility Mutation same as Rm7096; antibiotic marker different	86, 93
Rm7095	Rm1021 *exoR*::Tn*5*	Overproduction of succinoglycan Loss of motility	86
Rm7094	Rm1021 *exoB*::Tn*5*	Succinoglycan biosynthesis abolished	80
Rm8341	Rm1021 *exoZ*::Tn*5*	Succinoglycan lacks acetyl modification	81
Rm7154	Rm1021 *exoH*::Tn*5*	Succinoglycan lacks succinyl modification	119
Rm7445	Rm1021 *exoK*::Tn*5*	Reduction of cleavage of succinoglycan to LMW form	83
Rm7013	Rm1021 *exsH*::Tn*5*	Reduction of cleavage of succinoglycan to LMW form	83
Rm7014	Rm1021 *exoK*::Tn*5-233* *exsH*::Tn*5*	Reduction of cleavage of succinoglycan to LMW form	83

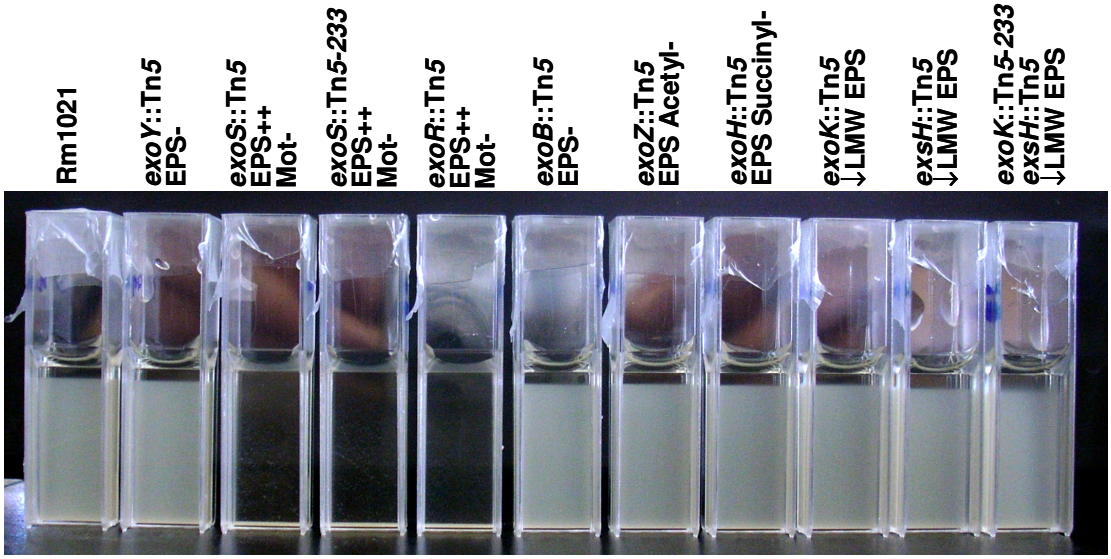


Figure 3-1 Screening of exopolysaccharide mutants for differences in rate of sedimentation. Each of these mutants is a derivative of the parent strain Rm1021. The location of the transposon is indicated in each case along with the relevant phenotype. EPS= succinoglycan, Mot= Motility. +/- = gain or loss of each phenotype. Cultures grown to late exponential phase in LB_{MC} and transferred into cuvettes. Incubated without shaking for 24 hours. Repeated experiments gave the same result

To show that the increased rate of sedimentation seen in the *exoS* mutant was due to aggregation of the cells, Rm1021 and the *exoS* mutant were compared by phase contrast microscopy (Fig 3-2). The *exoS* mutant forms small aggregates with the majority of the cells aligned laterally along their longest surface, side-by-side. The Rm1021 cells do not form any aggregates and remain as single cells (Fig. 3-2).

The *exoS* and *exoR* mutants were originally isolated from a transposon mutagenesis screen [86] and both have increased succinoglycan production and downregulation of flagella synthesis resulting in a loss of motility [86, 93]. At the beginning of this study the *exoS* mutant was better characterised than the *exoR* mutant, and it was therefore chosen to be the focus of the rest of the study. However it has been recently shown that the similarities in the phenotype of the *exoS* and the *exoR* mutants are due to ExoR acting as an inhibitor of ExoS [95].

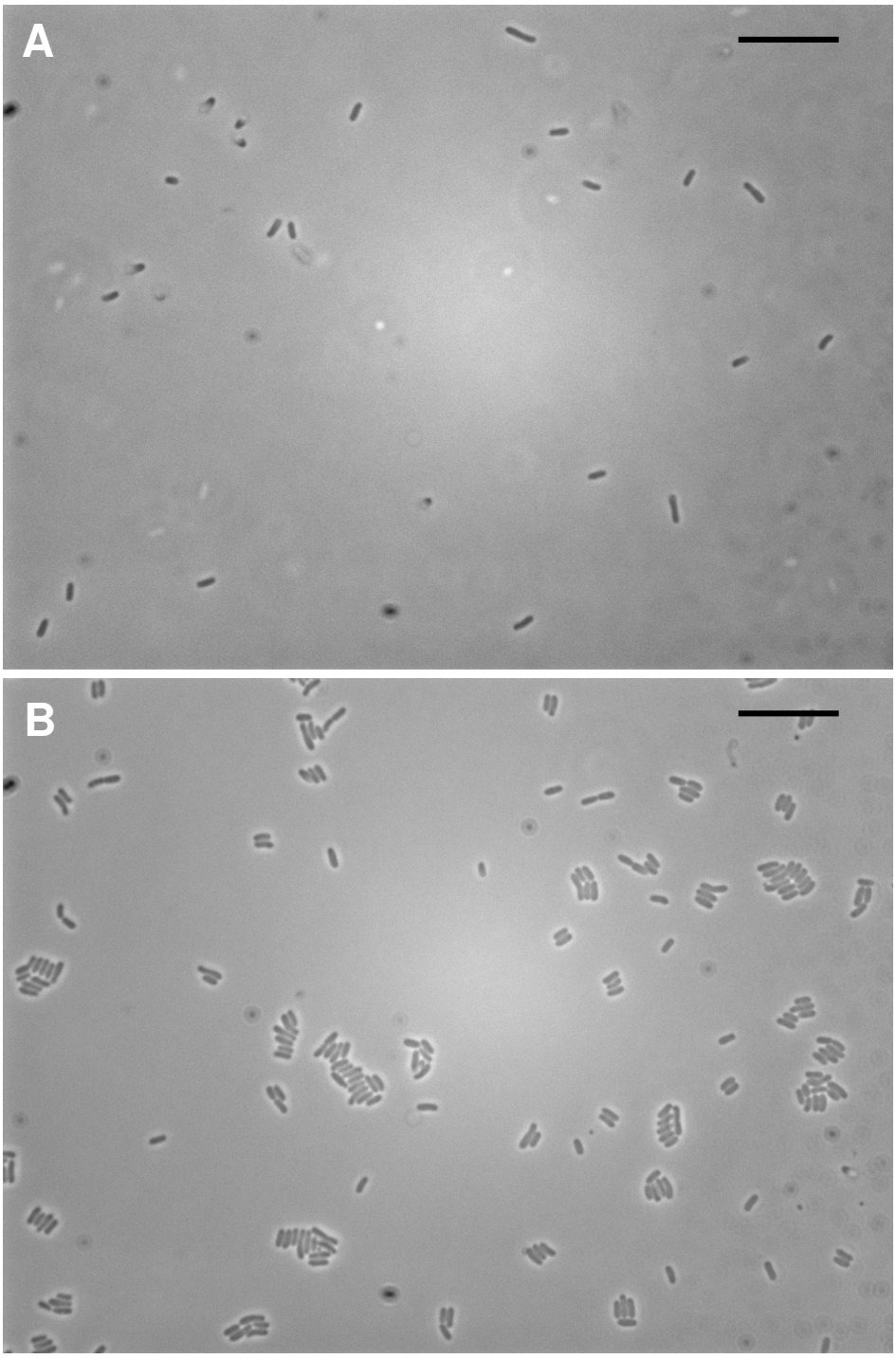


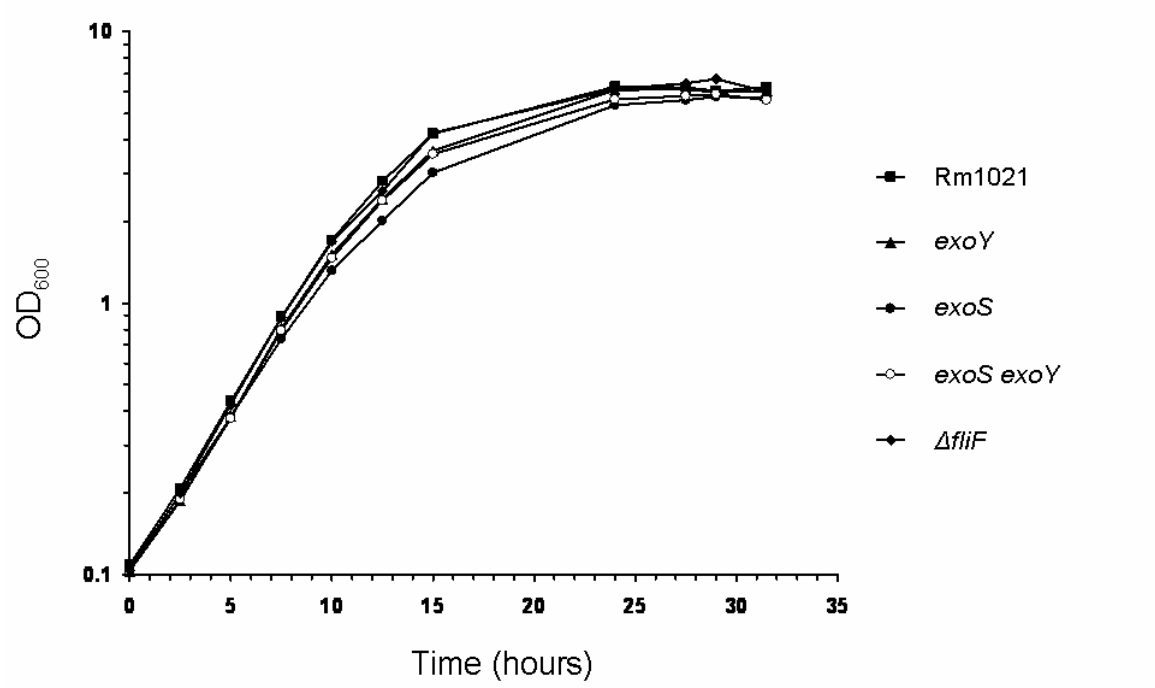
Figure 3-2. Phase contrast microscopy of Rm1021 and the Rm1021 *exoS* mutant. Cultures were grown to late exponential phase and imaged using phase contrast microscopy. A. Rm1021. B. *exoS* mutant. Bars=10µm.

3.2. Enhanced aggregation in the *exoS* mutant is due to overproduction of succinoglycan

From the initial screen and subsequent microscopy it remained unclear what was causing the increased aggregation in the *exoS* mutant. ExoS is the sensor histidine kinase of a two component regulator that may be affecting the transcription of a number of genes [92-95]. It is known that the *exoS* mutant, as well as overproducing succinoglycan, also has reduced motility due to the downregulation of the synthesis of flagella [93]. If succinoglycan was the cause of the *exoS* phenotype it was then unclear as to why the parent strain Rm1021 and the mutant strains capable of producing succinoglycan did not appear to differ from the strains incapable of producing succinoglycan, which all remained as stable, turbid cultures (Fig. 3-1). Therefore, to distinguish between the phenotypes of the *exoS* mutant as the causal mechanism behind the aggregation, three mutants were utilised. An *exoSexoY* double mutant was constructed to test whether the overproduction of succinoglycan is responsible for the observed phenotype of the *exoS* mutant and a Δ*fliF* mutant was used to investigate if loss of flagella alone could result in aggregation. These strains were screened and observed for sedimentation and aggregation.

3.2.1. Characterisation of the mutants

To ensure that all of the strains had equal growth rates and therefore comparable cell numbers after growth, OD_{600} and colony forming units were measured (Fig 3-3). All of the mutants had similar growth rates, approaching late exponential phase after 15 hours of growth at 30°C. OD_{600} was matched by an increase in CFU ml^{-1} in all strains (Fig. 3-3).



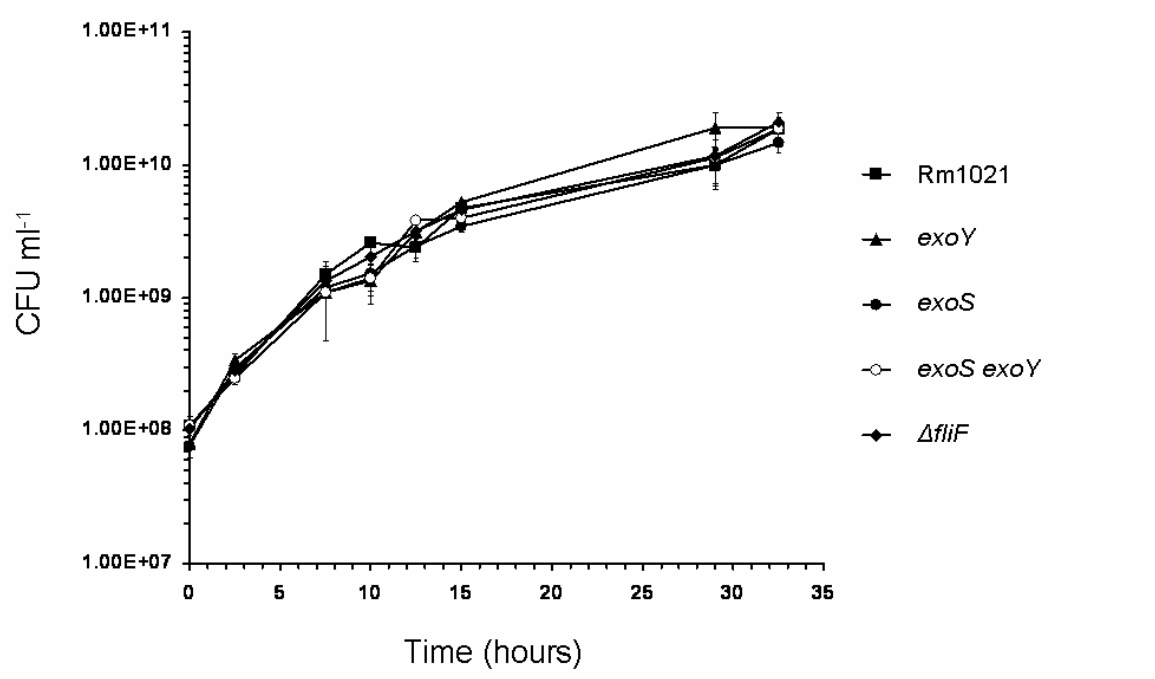


Figure 3-3 Growth of Rm1021 and derivative mutant strains in LB_{MC}. Cells were pre-grown from single colony for 24 hours at 30°C, inoculated into fresh medium and grown for time indicated at 30°C. At each time point OD_{600} (A) and colony forming units per ml (B) were assessed. Graphs are representative experiments. Error bars in B represent standard deviation. Experiments repeated gave the same result.

Loss of motility is one of the phenotypes of the *exoS* mutant [93] and so the motility of all five strains was compared by monitoring how the bacteria spread through soft LB_{MC} agar (0.3% w/v agar). Also, the Δ*fliF* mutant was previously constructed (Marlow, V and Ferguson, G unpublished data) but had not been fully characterised. Figure 3-4 shows that the *exoS*, *exoSexoY* and Δ*fliF* mutants formed only a small colony at the point of inoculation and did not spread through the medium at all, suggesting that all were non-motile (Fig. 3-4A). The parent strain Rm1021 and the *exoY* mutant are motile, with both spreading through the medium forming larger diffuse colonies. However, even after 48 hours the colony had not spread that far (~1cm) from the point of inoculation. All strains were observed by phase contrast microscopy in exponential phase and confirmed the motility results seen in the soft LB_{MC} agar plates. However from this microscopy it was apparent that even in the motile strains (Rm1021 and Rm1021 *exoY* mutant) few of the cells were actually motile.

To show that the Δ*fliF* mutants' loss of motility was due to loss of flagella (rather than paralysis of the flagella or loss of chemotaxis) the cells were imaged by TEM (Fig. 3-4B). In contrast to the parent Rm1021 the Δ*fliF* mutant was completely devoid of flagella. In the parent strain TEM flagella presumably shorn from the cells by preparation were visible, but were completely absent in the Δ*fliF* mutant. Once again however it could be seen that in the parent strain Rm1021 not all of the strains were flagellated. This data together suggests that while S. meliloti Rm1021 Δ*fliF* mutant is totally incapable of producing flagella, the parent strain is not producing flagella or as motile as perhaps would have been anticipated.

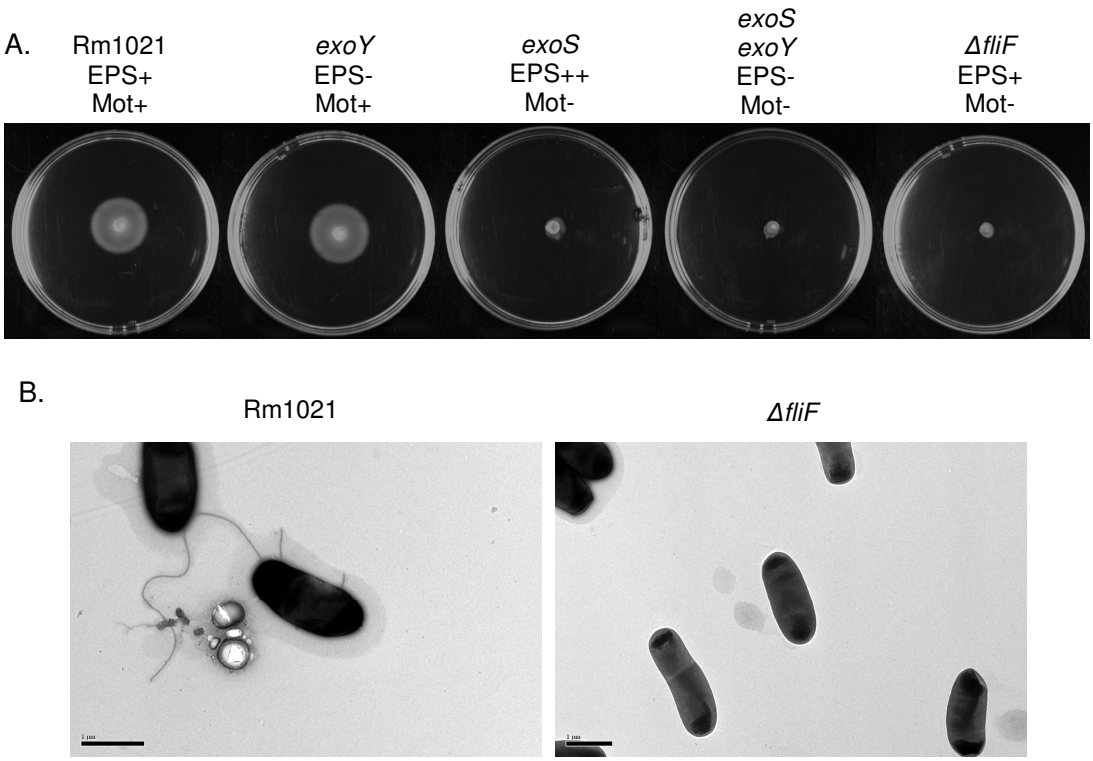


Figure 3-4. Motility of Rm1021 and derivative mutants. (A) Mutants were assessed for motility by inoculating late exponential phase cultures onto LB_{MC} 0.3% (w/v) soft agar plates and incubating at 30℃ for 48 hours. B. TEM images of Rm1021 and Δ*fliF* mutant show that unlike the parent the Δ*fliF* mutant is devoid of flagella. Bars= 1μm. Identity of strains indicated above image. repeated experiments gave similar results.

Cell size was measured by measuring cells from TEM images. All strains had similar lengths and diameters, suggesting that any differences in rates of sedimentation were not due to differences in cell size (Fig. 3-5).

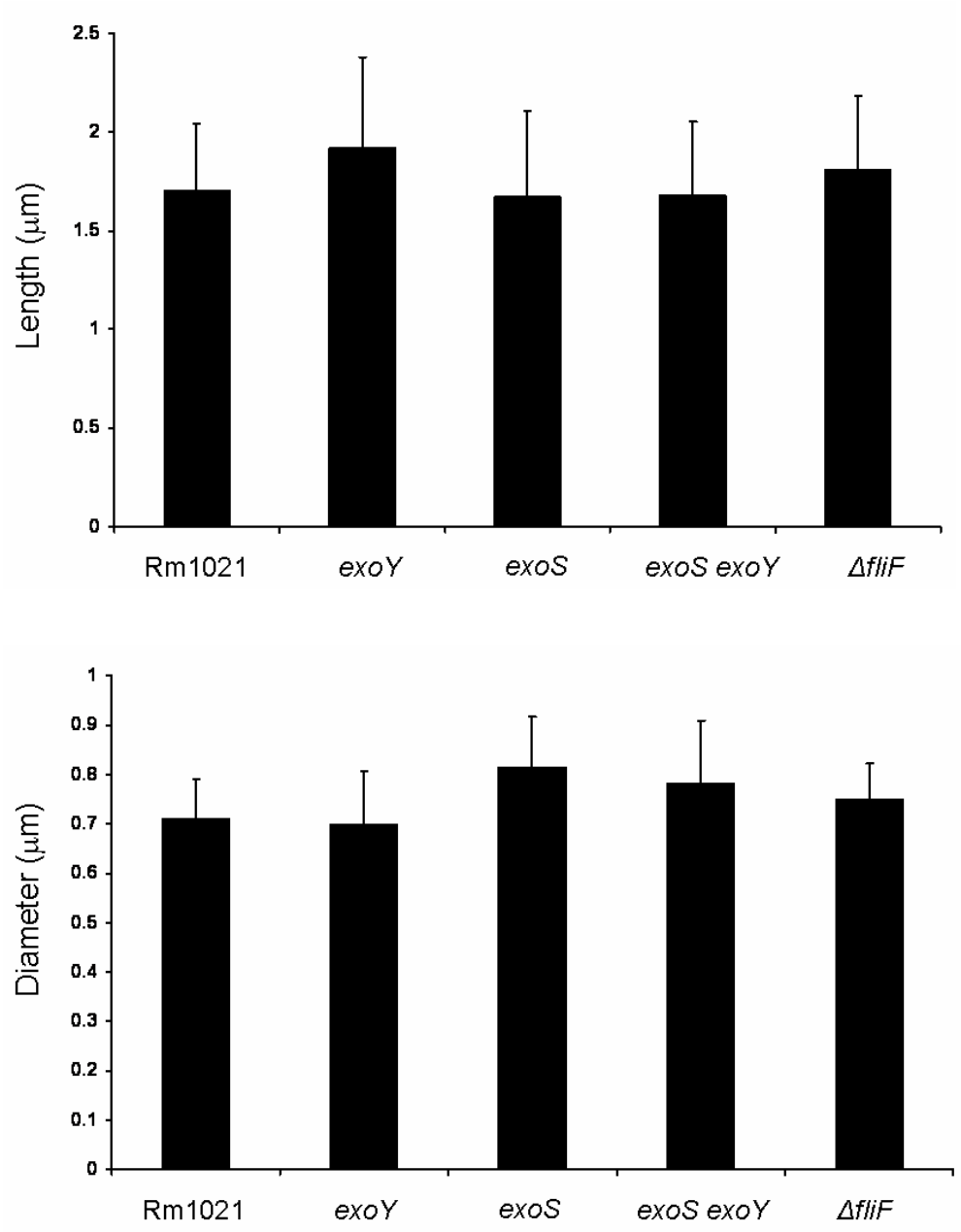


Figure 3-5. Average cell length and diameter. Cells were grown to late exponential phase and imaged using TEM before having cell lengths (A) and widths (B) measured. Error bars represent standard deviation.

To determine the relative amounts of succinoglycan being produced by the strains, polysaccharide was precipitated from the supernatant of the cultures by cetrimide and measured by the anthrone-sulphuric acid assay. Glucose was used to generate a standard curve. The amount of cetrimide-precipitable polysaccharide from

each of the supernatants is shown in Table 3-2. As found in previous studies the *exoS* mutant is overproducing succinoglycan [86]. All other strains tested do not appear to be producing any succinoglycan, or are producing very low levels in LB_{MC} (Table 3-1).

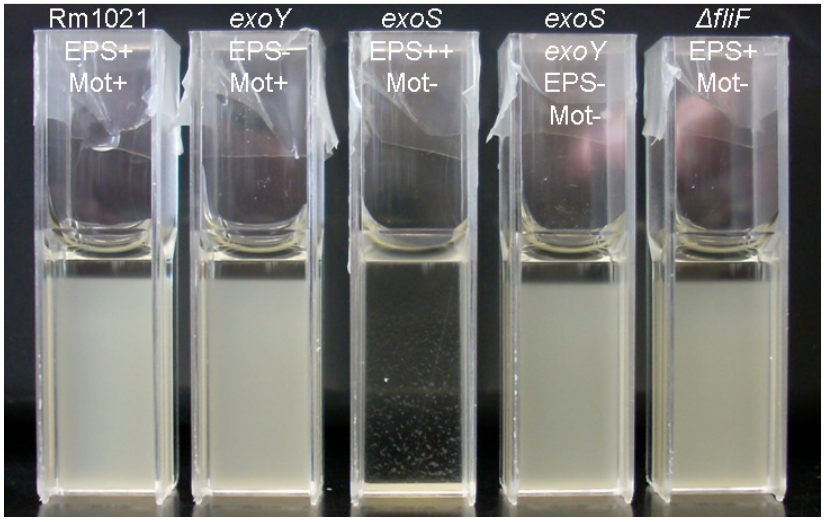
Table 3-2. Production of succinoglycan measured by the anthrone-sulphuric acid assay. Polysaccharide was precipitated from supernatant by Cetrimide after growth to late exponential phase in LB_{MC}. Experiments were repeated with the same result.

	Average μg glucose equivalents OD_{600}^{-1} ml^{-1}	Standard deviation
Rm1021	0.07	± 0.15
exoY	0.15	± 0.22
exoS	50.78	± 16.54
exoSexoY	0.00	± 0.11
Δ*fliF*	0.31	± 0.06

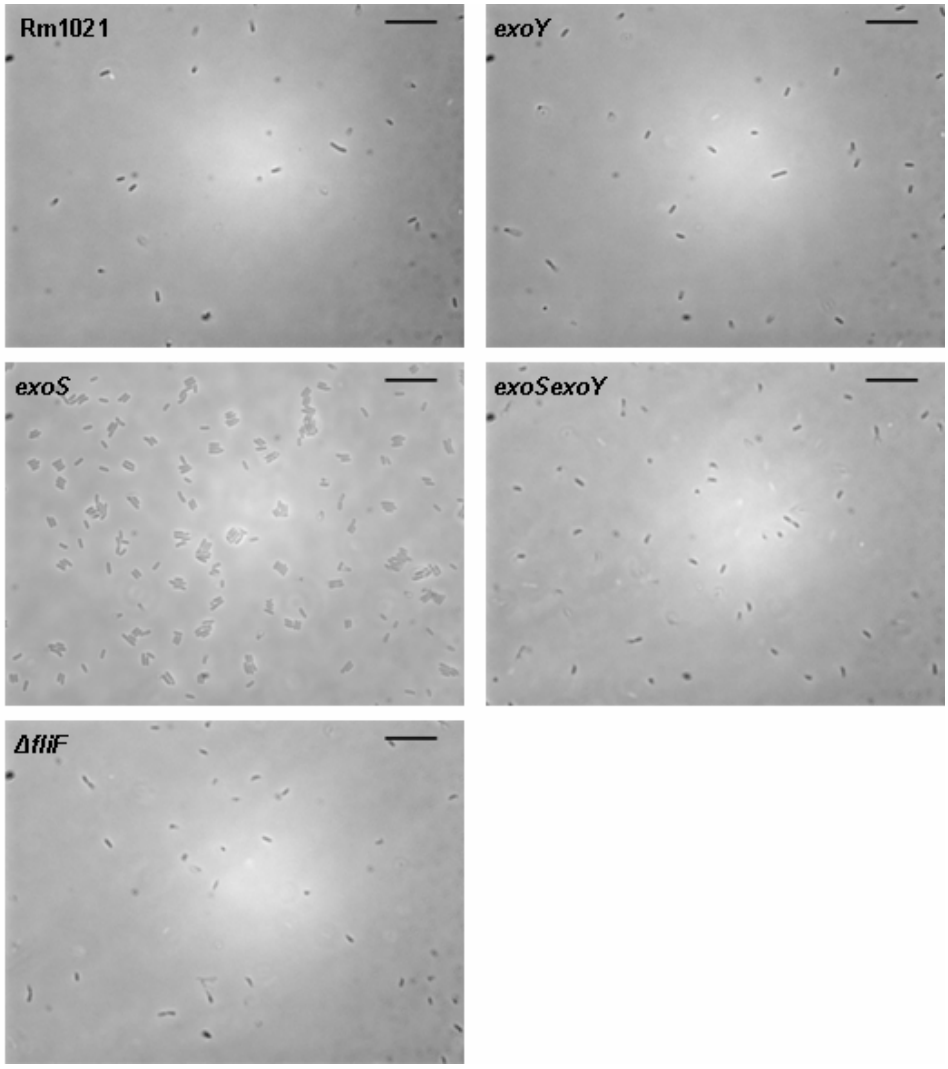
3.2.2 Only the exoS mutant has enhanced sedimentation

To find if the *exoY*, *exoSexoY* or Δ*fliF* mutants were aggregating, the strains were grown to late exponential phase, transferred into cuvettes and left without shaking for 24 hours. The *exoS* mutant was the only strain that formed aggregates resulting in sedimentation (Fig 3-6). All other strains remain turbid as stable dispersions (Fig 3-6). The same results were observed for stationary phase cultures. The sedimentation of the strains was monitored by measuring OD_{600} of the upper phase of the cuvette during static incubation. The *exoS* mutant was shown to sediment in less than ten hours whereas the other strains took over 120 hours to sediment to a similar level (Fig 3-6). CFU's of *exoS* cells redispersed after sedimentation showed the cells were still viable (~3×10^9 cells ml^{-1} before and after sedimentation).

A.


Rm1021
EPS+
Mot+
exoY
EPS-
Mot+
exoS
EPS++
Mot-
exoS
exoY
EPS-
Mot-
ΔfliF
EPS+
Mot-

B.


Rm1021
exoY
exoS
exoSexoY
ΔfliF

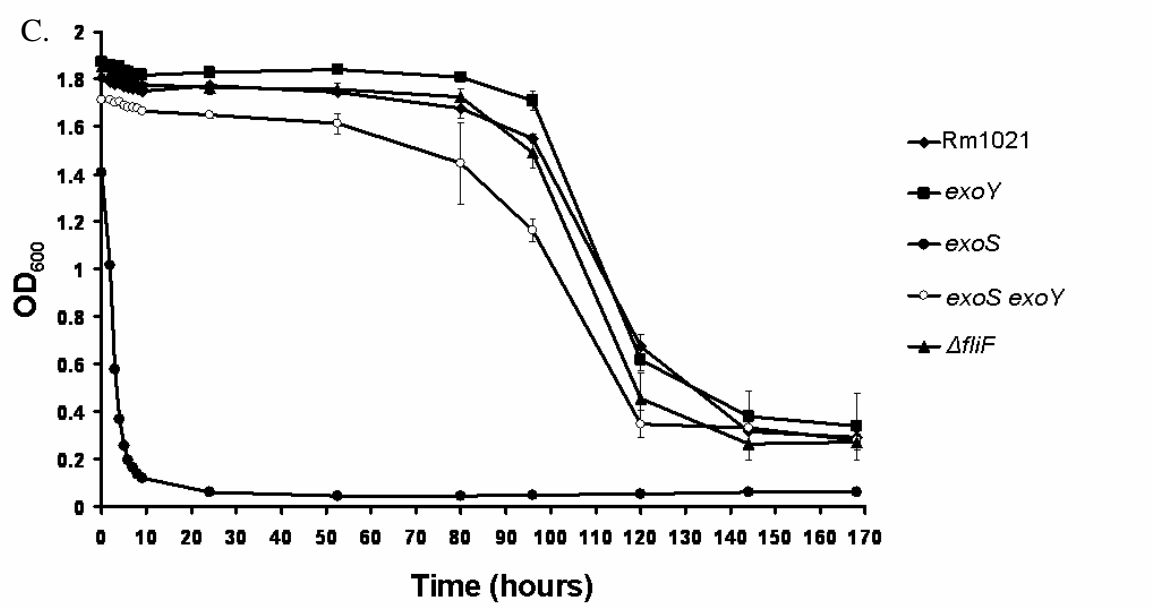


Figure 3-6. Sedimentation rate of Rm1021 and mutants strains. A. Strains were grown to late exponential phase, transferred into cuvettes and incubated without shaking for 24 hours. The parent Rm1021 and *exoY*, *exoSexoY* and Δ*fliF* mutants are all stable turbid dispersions. The *exoS* mutant has sedimented, leaving a clear upper phase. EPS= succinoglycan, Mot= Motility. B. Phase contrast microscopy of late exponential phase cultures. The *exoS* mutant forms small aggregates with cells aligned laterally. The other strains are all single cells with no aggregation apparent. Bars=10µm. C. Rate of sedimentation of the strains monitored by change in OD_{600} of the upper phase. Error bars represent standard deviation. All experiements were repeated with the same result.

3.2.3 Adding exoS supernatant to all strains results in an exoS phenotype

It therefore appeared that the overproduction of succinoglycan was the cause of the enhanced aggregation and sedimentation in the *exoS* mutant. While the *exoSexoY* mutant showed that succinoglycan was essential for the aggregation to occur it seemed possible that the loss of flagella in the *exoS* mutant could also be necessary for the succinoglycan-driven aggregation. This would not be seen in the Δ*fliF* mutant in the above experiments due to the low production of succinoglycan by the Δ*fliF* mutant in LB_{MC}.

To investigate this further succinoglycan was added exogenously to the parent strain and other mutants. A preparation of succinoglycan was made by taking advantage of the fact that extracellular polysaccharides are secreted into the medium and can therefore be separated from the cells by centrifugation. A culture of the *exoS* mutant was centrifuged and the resulting supernatant was filter sterilised. Absence of cells of the *exoS* mutant was ensured by carrying out a serial dilution and inoculating a LB_{MC} plate. Late exponential phase cultures of each strain were then centrifuged, the supernatant discarded and the pelleted cells dispersed in *exoS* supernatant containing succinoglycan (Fig. 3-7). After 24 hours incubation without shaking all the strains had sedimented in the presence of supernatant from the *exoS* mutant (Fig. 3-7). Phase contrast microscopy of the strains in the presence of *exoS* supernatant showed that the cells were forming similar aggregates as the *exoS* mutant, as can be seen for Rm1021 in figure 3-8. Aggregation still occurred in Rm1021 even though motile cells were present, suggesting that absence of flagella is not necessary for aggregation to occur in the presence of succinoglycan. Cells were observed a few minutes after adding the supernatant and the cells were already aggregating.

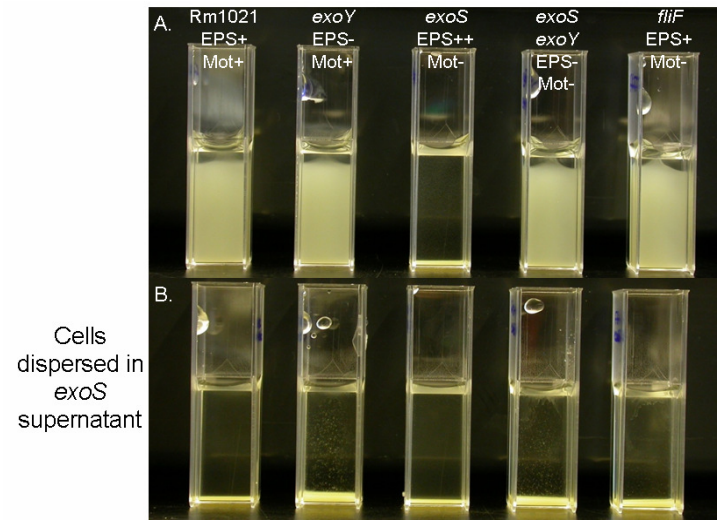


Figure 3-7. Addition of exogenous succinoglycan in the form of *exoS* supernatant gives all strains an *exoS* phenotype. Cells were grown to late exponential phase either A. incubated without shaking for 24 hours. B. strains were pelleted by centrifugation and dispersed in filter sterilised *exoS* supernatant, before incubating without shaking for 24 hours. Experiments repeated with the same result.

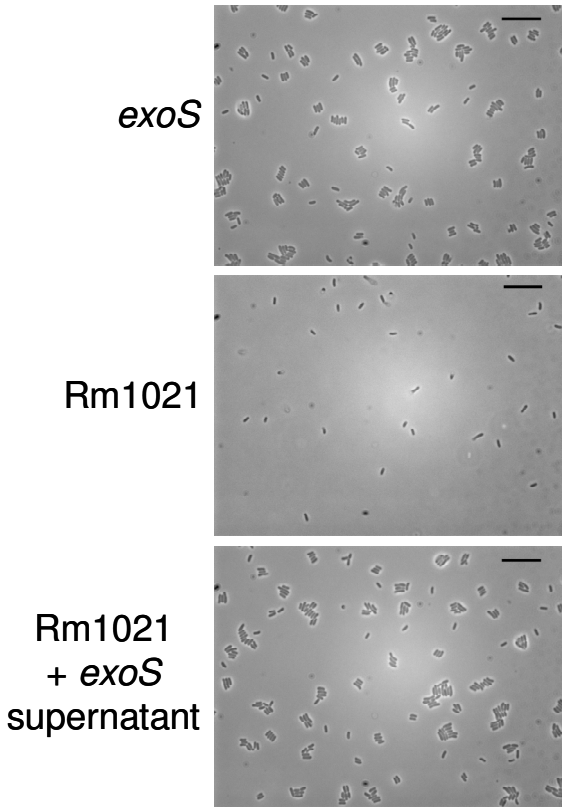


Figure 3-8 Phase contrast microscopy of addition of *exoS* supernatant to Rm1021 cells. Cells were grown to late exponential phase and imaged using phase contrast microscopy. Rm1021 cells are not aggregating, whereas the *exoS* mutant forms aggregates. Addition of *exoS* supernatant to Rm1021 results in an *exoS* phenotype. Bars=10μm. Experiments repeated with the same result.

A further preparation of succinoglycan was made from the *exoS* supernatant. Cetrimide (hexadecyl-trimethyl-ammonium bromide) can be used to precipitate succinoglycan due to the acidic groups on the succinoglycan polymer [68]. The precipitate was pelleted by centrifugation, the succinoglycan-free supernatant was removed, and the pellet was resuspended in fresh LB_{MC}. All the strains were then dispersed in both the succinoglycan-free supernatant and the cetrimide-purified succinoglycan (Fig. 3-9). All strains had an *exoS* phenotype in the presence of the resolubilised cetrimide-purified succinoglycan with enhanced sedimentation. The strains remained stable, disperse cultures in the presence of succinoglycan-free *exoS* supernatant (Fig 3-9). Together these results indicate that overproduction of succinoglycan is responsible for the aggregation phenotype of the *exoS* mutant.

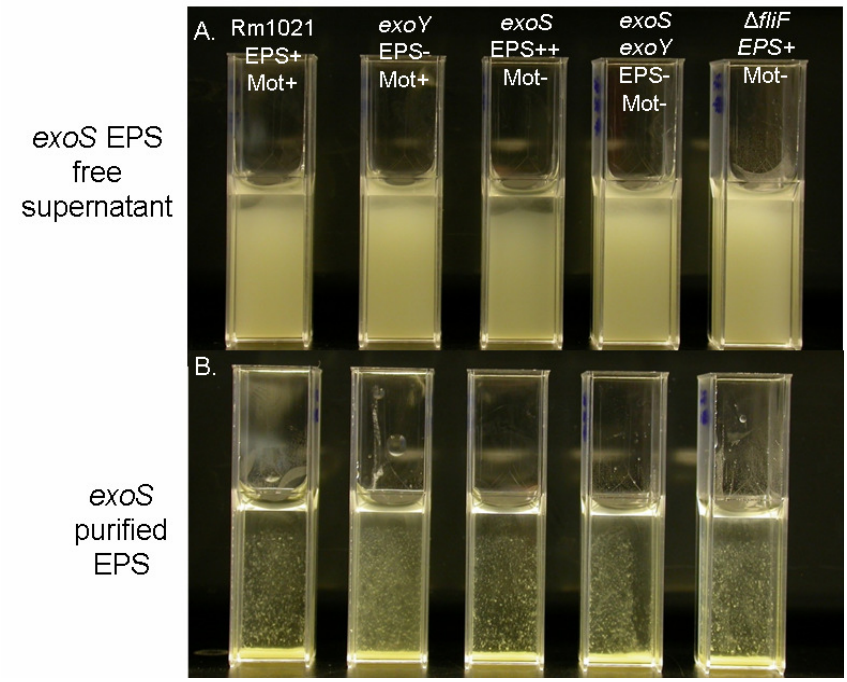


Figure 3-9 Addition of cetrimide-purified succinoglycan to all strains results in an *exoS* phenotype. Succinoglycan was precipitated from exoS supernatant by addition of Cetrimide. The precipitate was pelleted and the supernatant collected. The pellet was then resolubilised in fresh LBMC. Rm1021 strains were then dispersed in these two solutions. Succinoglycan-free supernatant resulted in stable turbid cell dispersions whereas addition of Cetrimide purified-succinoglycan resulted in sedimentation. EPS= succinoglycan, Mot= motility, +/- indicates gain or loss. Experiment repeated with the same result.

3.2.4 Loss of the succinyl or acetyl modifications from succinoglycan does not alter the exoS phenotype

Having shown that the production of succinoglycan was responsible for the aggregative *exoS* phenotype, the *exoZ* and *exoH* mutations were introduced into the *exoS* background. The *exoZ* mutation means that the resulting strain is unable to acetylate the succinoglycan while the *exoH* mutation results in a loss of the ability to add the succinyl group [85]. Both mutations can also result in further alterations to the polysaccharide; most noticeably an alteration in the production of the LMW succinoglycan by cleavage of the HMW form by the glycanases ExoK and ExsH [85].

Figure 3-10 shows that static incubation of the *exoSexoZ* and *exoSexoH* mutants resulted in enhanced sedimentation of the cells compared to the parent Rm1021. This is the same as seen for the *exoS* mutant (Fig. 3-10). The loss of either the acetyl or succinyl modification does not appear to affect the aggregation of the *S. meliloti* cells in the presence of succinoglycan (Fig. 3-10).

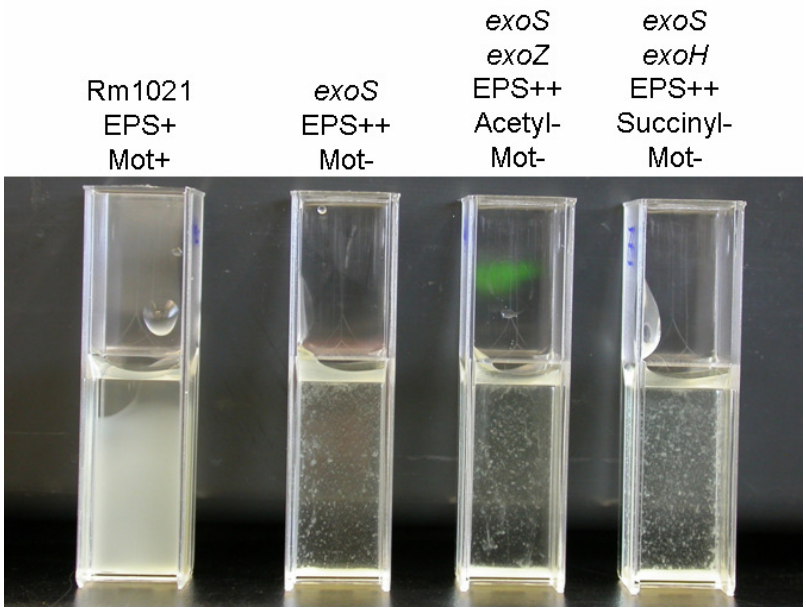


Figure 3-10 Sedimentation of the *exoSexoZ exoSexoH* mutant is faster than the parent Rm1021 but the same as the *exoS* mutant. Cultures were grown to late exponential phase and observed for sedimentation after 24 hours. Experiment was repeated with the same result.

3.3. Succinoglycan induced aggregation is driven by a physicochemical mechanism

Succinoglycan had been shown to be the causal factor for the aggregation and resulting enhanced sedimentation of the *exoS* mutant. Two possible hypotheses were formulated for how succinoglycan may induce aggregation: (i) the polysaccharide physically acts on the cells via either a bridging or depletion attraction mechanism,

(ii) the succinoglycan was acting as a signal to induce changes in the cell surface which resulted in an increase in aggregation of the cells. In the parent Rm1021 succinoglycan is known to be produced in a high molecular weight (HMW) form consisting of thousands of repeats of the subunit and a low molecular weight (LMW) form that is made up of monomers, dimers and trimers of the repeat unit [70]. The LMW form has been shown to act as an essential signal in the symbiosis by initiating the formation of the infection thread through which the bacteria invade the host plant cells [69]. It therefore seemed possible that the LMW form could also act as a signal to alter the phenotypes of the cells.

To find out if the *exoS* mutant produced a HMW and LMW form of succinoglycan a crude preparation of succinoglycan was prepared by dialysis of supernatant from an *exoS* mutant followed by size exclusion chromatography. Blue dextran and glucose were used to determine the void volume and included volume respectively. Analysis of the fractions collected from the chromatography showed that the *exoS* mutant produces succinoglycan consisting of a large HMW peak and a smaller LMW peak (Fig. 3-11).

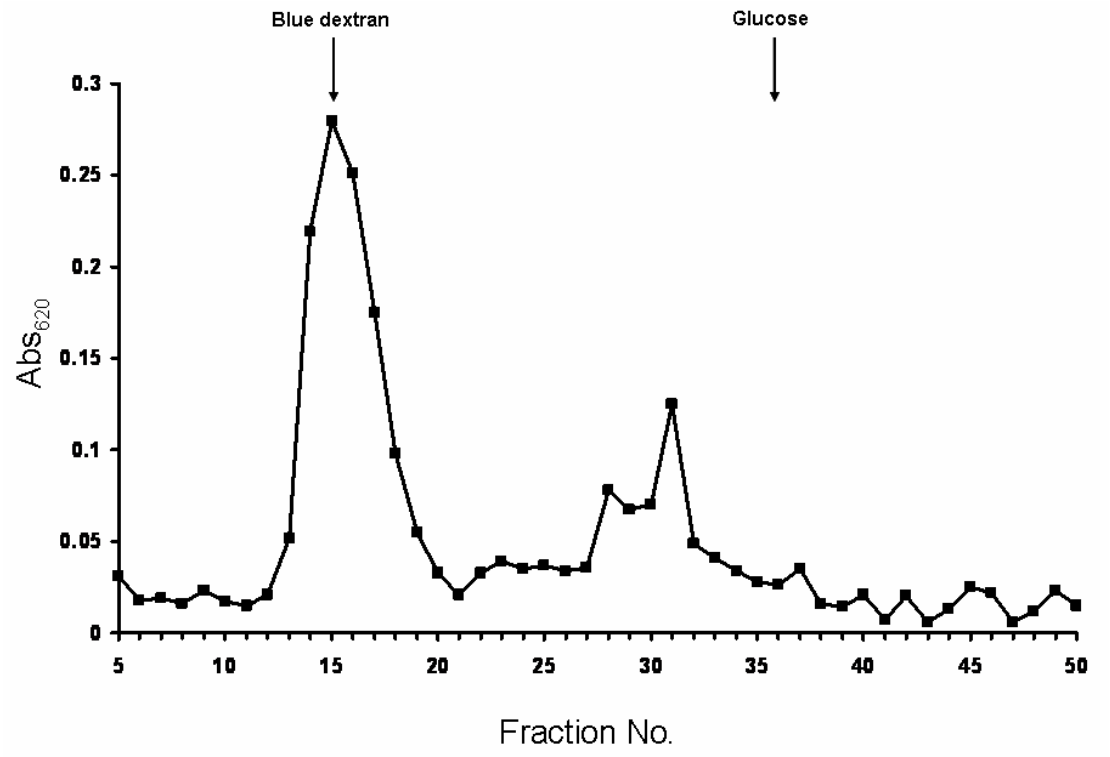
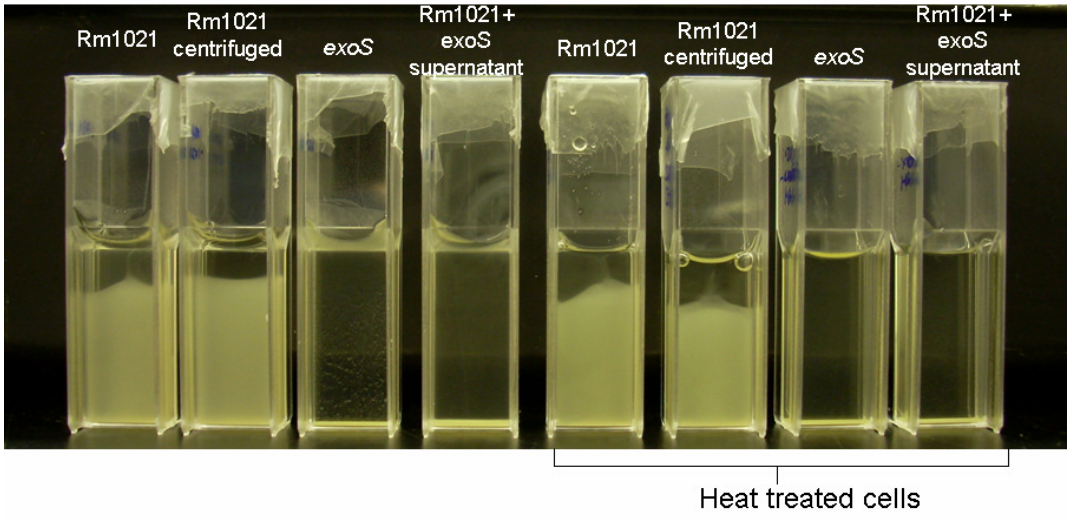


Figure 3-11. Sepharose 4B Gel filtration chromatography of succinoglycan from *exoS* supernatant. Arrows indicate fractions at which blue dextran (approximately 2MDa) and glucose eluted. Experiment was repeated with the same result.

To discern whether the succinoglycan was acting as a signal Rm1021 cells were heat treated at 60°C for one hour, which reduced the viability of the cells from approximately 1.9×10^{9} cells ml^{-1} post incubation to less than 500 cells ml^{-1} after heat treatment. The non-viable heat treated Rm1021 cells were then dispersed in filtered *exoS* supernatant and were found to have an *exoS* aggregative phenotype as found in Rm1021 cells without heat treatment (Fig. 3-12). Rm1021 cells dispersed in *exoS* supernatant were also found to be aggregating when imaged by phase contrast microscopy (Fig. 3-12). Images were taken immediately after the cells had been dispersed in *exoS* supernatant and it was found that the cells had already started aggregating (Fig. 3-12).

A.



B.

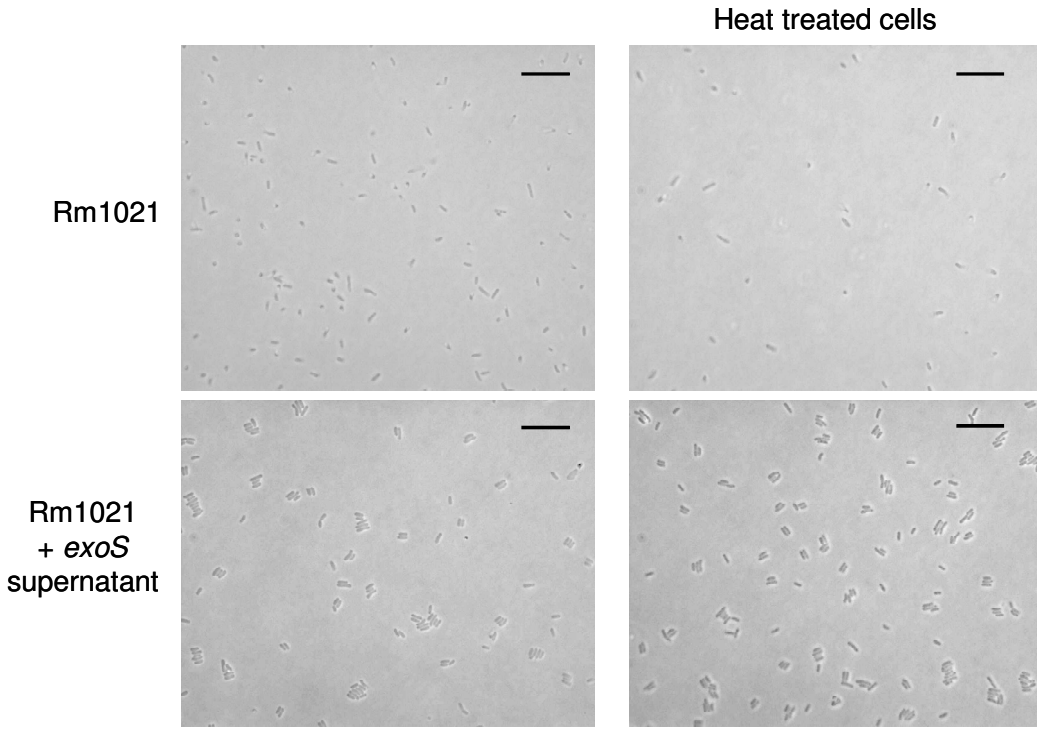


Figure 3-12. Heat treated, non-viable cells have an *exoS* phenotype in the presence of *exoS* supernatant. A. Cuvettes on the right indicate heat treated cells. Rm1021 cells dispersed in exoS supernatant have sedimented as have the viable cells on the left. Rm1021 centrifuged is a control to show that centrifugation of the cells does not alter sedimentation after heat treatment. B. Microscopy of Rm1021 with and without the presence of *exoS* supernatant before and after heat treatment. Bars=10µm. Experiments were repeated with the same result.

3.4. Discussion

Polysaccharides produced by bacteria are hugely diverse but in many species it has been shown that the production of these polysaccharides is concomitant with the formation of aggregates and biofilms [53]. Before this study, aggregation in *S. meliloti* by succinoglycan had not been demonstrated. The aim of this part of the study was to identify whether succinoglycan had a role to play in aggregation of *S. meliloti* Rm1021 and whether various Rm1021 mutants with altered succinoglycan biosynthesis affected this aggregation. Observation of the exopolysaccharide mutants subsequent to culture showed that some of the strains sediment at different rates. Screening isolated two succinoglycan overproduction mutants, *exoS* and *exoR* that have increased rate of sedimentation due to aggregation of the cells by succinoglycan.

3.4.1 The exoS mutant forms aggregates due to increased production of succinoglycan

The *exoS* and *exoR* mutants both have increased sedimentation and aggregation compared to the parent Rm1021. Both of these mutants have been shown previously to share phenotypic characteristics [86, 93]. At the beginning of this study little was known about ExoR and the focus was therefore on the *exoS* mutant which was better characterised. However since then it has become clear that ExoR is a negative regulator of ExoS, hence the reason for the similarities in phenotypes of the two mutant strains [94, 95].

ExoS is a two component regulator which acts in concert with the response regulator ChvI to regulate the transcription of a number of genes [92]. The *exoS* mutation is a gain of function mutant and so ExoS is constitutively active in this

strain resulting in the upregulation of succinoglycan biosynthesis but downregulation of motility [86, 92, 93]. The increased aggregation in this mutant could therefore have been a result of the polysaccharide, a reduction in repulsion between the cells due to loss of flagella or a synergistic effect of the two.

To find if the increased succinoglycan was the cause of the *exoS* aggregative phenotype an *exoY* mutation, which results in the loss of the ability to produce succinoglycan, was introduced into the Rm1021 *exoS* background. The *exoSexoY* mutant did not aggregate supporting the hypothesis that succinoglycan was responsible for aggregation of the *exoS* mutant. Additional evidence was provided by the fact that dispersing *S. meliloti* cells in succinoglycan results in aggregation.

Further to this, the addition of succinoglycan to strains with flagella resulted in aggregation, as well as in those strains that do not have flagella. This therefore suggests that it is solely the overproduction of succinoglycan in the *exoS* mutant which is responsible for the aggregation. However this should be treated with caution. Observation of motility of the cells showed that in a culture of the parent strain Rm1021 only a small percentage of the population were actually motile, which only gave a small amount of outgrowth over 48 hours as shown by growth of the cells on soft agar LB_{MC}. Further direct observation of flagella by TEM showed that in the parent strain Rm1021 many of the cells did not have flagella. However this could be due to the preparation procedure and removal of the flagella by centrifugation. It therefore is unclear to what extent the cells of a culture of Rm1021 are flagellated. The role of flagella and motility in the process of aggregation by succinoglycan is therefore unclear. This could be further investigated by using a dye that binds to flagella when imaging aggregates to see if cells outside of aggregates

are flagellated. Interestingly, when succinoglycan is added to the strains with flagella, while many cells aggregate there are some that continue to be motile and do not become aggregated. In the case of *S. meliloti* it therefore seems possible that while the presence of flagella does not necessarily reduce aggregation *per se*, cells that are highly motile are able to remain outside of aggregates. This point will be returned to in chapter five, where the effect of succinoglycan on aggregation of *E. coli* MG1655 will be investigated.

Transfer of the *exoZ* and *exoH* mutations into the *exoS* background, which results in loss of the acetyl and succinyl modifications of the succinoglycan respectively, did not affect cell aggregation or sedimentation. Absence of either the acetyl or succinyl groups can also alter other aspects of succinoglycan. For example the loss of the succinyl group decreases the production of LMW succinoglycan, whereas absence of acetyl groups leads to an increase in LMW succinoglycan [85]. This is due to these groups modulating the activity of the glycanases ExoK and ExsH [85]. Further, loss of the succinyl modification can also increase the stiffness of the polymer, perhaps as a result of stabilisation of the helix [73]. However none of these effects appeared to influence the ability of succinoglycan to cause aggregation of *S. meliloti*.

3.4.2 The parent strain Rm1021 does not aggregate in LB_{MC} due to a lack of succinoglycan production

While the *exoS* and *exoR* mutants overproduce succinoglycan and have enhanced aggregation as a result, it was interesting that in LB_{MC} the parent strain Rm1021, which is capable of producing succinoglycan, does not appear to aggregate at all. There was no apparent difference between the sedimentation rate of

the mutants that are unable to produce succinoglycan (*exoY* and *exoB*) and the parent strain Rm1021 (and Rm1021 mutants still able to produce succinoglycan). All the strains show some sedimentation due to gravity, but they remain dispersed stable turbid cultures for several days before all the cells finally sediment to the bottom of the cuvette, in contrast to the *exoS* mutant which sediment in a few hours. This lack of aggregation appears to be due to the fact that in LB_{MC} the production of succinoglycan by Rm1021 is extremely low. This is supported by the similarity in the dry colony morphology of Rm1021 and the *exoY* mutant on LB_{MC} agar. The lack of succinoglycan biosynthesis by Rm1021 in complex media could be a result of domestication in the laboratory. It appears that in laboratory cultures exopolysaccharides are either not expressed or the ability to produce them may be lost since this does not impair survival or viability as it may do in nature where their role in aggregation is essential to survival [38]. In Rm1021 the ability to produce succinoglycan has not been lost and can still occur in certain minimal media (see chapter 5), but is reduced in LB_{MC}.

It is also perhaps surprising that in LB_{MC} the growth rate of the *exoS* mutant is similar to the other strains, considering that it is producing so much more polysaccharide. For example, the octasaccharide unit of the succinoglycan is assembled on polyprenyl lipid membrane carriers which are also used to construct LPS and peptidoglycan [91]. However, even though the *exoS* mutant appears to be constitutively overproducing succinoglycan compared to the parent, more polysaccharide can be made by this mutant when grown in minimal media conditions [86]. It is possible therefore that in LB_{MC} the rate of production of succinoglycan in the *exoS* mutant is not high enough to inhibit the use of the polyprenyl lipid membrane

carriers to synthesise the LPS and peptidoglycan molecules needed for growth and replication of the cells.

Therefore, succinoglycan can result in aggregation in *S. meliloti* Rm1021 but only when produced in high enough amounts. Cell-cell aggregation by extracellular polysaccharides has been demonstrated in other *Rhizobium* species [14, 15]. For example in *R. leguminosarum*, the production of extracellular fibrils was correlated to the formation of aggregates of cells forming caps on root hair tips [14, 15]. During the course of this study it has also been shown by others that *S. meliloti* can form biofilms, although these studies have focussed more on the attachment of the cells to a surface rather than cell-cell aggregation [95, 99, 132]. In this case it was also shown that the *exoS* mutant had enhanced biofilm formation [132] (see also chapter five).

3.4.3 Succinoglycan causes aggregation of S. meliloti by a physicochemical mechanism

The mechanism by which the enhanced levels of succinoglycan may be resulting in aggregation appears to be physicochemical rather than due to adaptation of the cell surface in response to the presence of succinoglycan. Heat treated cells of Rm1021 which are no longer viable still aggregate and sediment in the presence of exogenous succinoglycan. Further, addition of succinoglycan to Rm1021 cells appeared to result in aggregation of cells straight away as visualised by microscopy. This suggests that the cells are not adapting to the presence of succinoglycan, for example by production of a lectin, and a physical mechanism is more likely.

In summary therefore it appears that succinoglycan, as well as acting as a signal in the initiation of the symbiosis with the plant host [123], when present in high enough amounts can also aggregate *S. meliloti* Rm1021. In many of these studies the polysaccharide is ascribed a bridging role in the formation of aggregates. However in observing *S. meliloti* it became clear that there were several features which led to an evaluation of the mechanism by which succinoglycan may cause aggregation. Due to their dimensions bacteria can be modelled as colloidal dispersions and as such the forces that govern colloidal interactions will also have a role in bacterial cultures. The addition of polymers to colloidal dispersions can result in aggregation of the particles via two alternative mechanisms: bridging flocculation or depletion attraction. These two mechanisms will be further explored in the following chapter. The biological role of aggregation regulated by ExoS will also be investigated in chapter five.

Chapter 4

Aggregation *of S. meliloti* by succinoglycan is caused by depletion attraction

The enhanced aggregation of the *exoS* mutant is due to the overproduction of succinoglycan. In many bacterial systems polysaccharides make up a large component of aggregates and biofilms and as such it is thought the polysaccharides may provide the 'glue' by which cells are stuck together [7, 53]. To see if this was also the case in *S. meliloti* aggregation, the mechanism by which the presence of succinoglycan resulted in aggregation was investigated. The size of a *S. meliloti* bacterium (~ 0.8μm × 2μm) puts it in the colloidal range so that the physicochemical basis for this aggregation may be found in effects of polymers on colloidal dispersions. In the presence of polymers aggregation of colloidal particles commonly occur via two different mechanisms, bridging flocculation and depletion attraction. The difference lies in the interaction between the polymers and the particles. In the case of bridging the polymer adsorbs to the surface of different particles, which at high enough concentrations results in the polymer bridging between the different particles, drawing them together into aggregates (flocs). As mentioned, this is hypothesised to be the mechanism of aggregation in a number of bacterial-polysaccharide systems [7, 53]. An alternative mechanism, well known to physicists but largely ignored by biologists is known as depletion attraction. This occurs because the approach of a polymer to the particle surface results in a reduction in the

conformational entropy of the polymer and is therefore unfavourable. There is then a region next to the surface of a particle from which the polymer is effectively excluded, also known as a depletion zone. This results in an osmotic imbalance between the zone of depletion around the particle and the bulk volume. When two particles come closer together the zones of depletion overlap and due to the imbalance in osmotic pressure, solvent diffuses from the gap resulting in the particles being pushed together. Equivalently this arrangement also increases the configurational entropy of the polymers. As the depletion zones overlap the volume accessible to the polymer increases resulting in an increase in their entropy. This entropic effect is similar to what many biologists refer to as 'macromolecular crowding' [105]. Crowding or depletion attraction has been shown to drive the organisation of many cellular structures in eukaryotic cells [105, 108, 111]. More recently depletion attraction has been shown to play a role in bacterial aggregation in the presence of synthetic polymers and DNA [117, 118].

Bridging flocculation and depletion attraction differ in a number of ways that can be used to distinguish the two mechanisms. By observing the demixing or sedimentation of the cells in response to different concentrations of cells and polymers it is possible to discriminate between bridging and depletion. Bridging will be limited by polymer concentration as the cell concentration increases, whereas depletion will be enhanced at higher cell concentrations for a given polymer concentration as it is a crowding phenomenon.

4.1 The aggregation of the *exoS* mutant has characteristics of depletion attraction

The aggregation and resulting destabilisation of colloidal dispersions, as seen in the *exoS* mutant cultures, could have been due to either bridging flocculation or depletion attraction. As mentioned above the favoured hypothesis for the mechanism of bacterial aggregation by polysaccharides is bridging flocculation. However the *exoS* mutant had several characteristics which made depletion attraction the more likely hypothesis for this system.

4.1.1 Absence of polymer associated with exoS mutant aggregates is indicative of depletion attraction

Visual observation of the *exoS* cells by phase contrast microscopy showed that the cells were forming small aggregates (Fig. 3-2B and 4-1A), which led to the destabilisation and sedimentation of the cultures. One of the main features of bridging flocculation is that the polysaccharide adsorbs to the cells drawing the cells together into an aggregate, whereas in depletion the polymer would have to be non-adsorbing. The hypothesis that succinoglycan may not adsorb to the cells, and therefore may promote aggregation by depletion attraction, is first supported by the fact that there will be a repulsive electrostatic interaction between the net negative charge of the cell surface and succinoglycan which is an anionic exopolysaccharide. The acidic non-carbohydrate substituents pyruvate and succinate on succinoglycan will confer a negative charge at physiological pH [53, 68]. The cell surfaces of bacteria are also generally thought to have a net negative charge, although some species have been found that have a net positive charge [117, 133-135]. To gain an estimation of the net

charge on the surface of *S. meliloti* cells the electrophoretic mobility of the strains was measured by a zeta potential analyser. The cells were grown to late exponential phase and dispersed in PBS at pH7. The electrophoretic mobility for the different strains is given in table 4-1. All the strains used in the sedimentation screens in chapter three have negative values, indicating that the *S. meliloti* cell surface has a net negative charge and electrostatic repulsion would inhibit the polysaccharide adsorbing to the cells.

Table 4-1. Electrophoretic mobility of late exponential *S. meliloti* strains in PBS. Standard deviation is from triplicate samples measured for every experiment.

Strain	Electrophoretic mobility ($\mu m\ s^{-1}$)(V/cm) ± SD
Rm1021	-2.3 ± 0.11
exoY	-2.0 ± 0.04
exoS	-2.3 ± 0.15
exoSexoY	-2.2 ± 0.02
Δ*fliF*	-2.1 ± 0.04

Another factor that may be important in the interaction between the cell surface and the polysaccharide is the fact that succinoglycan is an exopolysaccharide which is secreted into the medium and can be washed away from the cells by centrifugation. Bacteria produce a number of extracellular polysaccharides. Exopolysaccharides such as succinoglycan are differentiated by the fact they have no permanent linker to the cell membrane [35]. This is in contrast to capsular polysaccharides which may be covalently bonded to the cell membrane and remain attached to the cells even after centrifugation [35]. By definition therefore exopolysaccharides are secreted into the medium when bacteria are grown in liquid

culture. Also, in the case of bridging flocculation the polysaccharide forms an extracellular matrix which can then be visualised by microscopy as a dense layer encasing the cells. However in the case of the *exoS* mutant no polysaccharide could be visualised around the aggregates (Fig. 4-1A). This was further supported by addition of calcofluor to the *exoS* cultures (Fig. 4-1B). Calcofluor is a fluorescent dye that binds to β-1, 4- glycosidic linkages and has been used to visualise the production of succinoglycan by Rm1021 on LB_{MC} agar plates when screening for succinoglycan mutants [131]. Addition of calcofluor to the *exoS* cultures therefore allowed visualisation of the succinoglycan in the medium. This showed that the fluorescently labelled succinoglycan appears to be dispersed throughout the medium (Fig. 4-1B). In particular the aggregates of cells were not calcofluor bright, as would be expected if succinoglycan was adsorbing to the cells and encasing the aggregates (Fig. 4-1B).

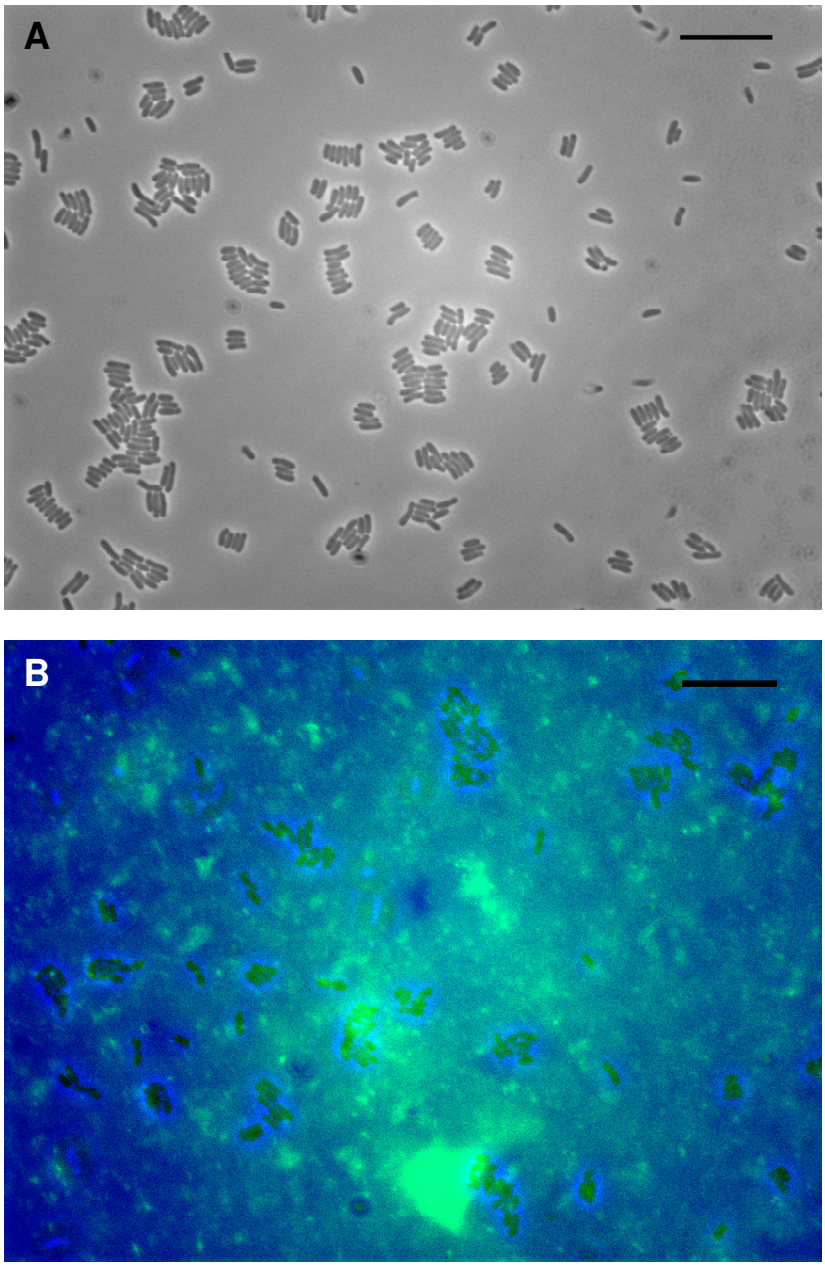


Figure 4-1. Microscopy of *exoS* aggregates. A. Phase contrast microscopy of aggregates formed by the *exoS* mutant after culture to late exponential phase. B. Microscopy showing distribution of succinoglycan in *exoS* mutant culture. Cells were imaged by phase contrast microscopy which was then overlaid with a fluorescence microscopy image that visualised calcofluor binding to succinoglycan. Bars= 10µm. Similar observations were made with repeated experiments.

4.1.2 Arrangement of cells in exoS mutant aggregates is consistent with maximisation of the depletion interaction

As well as polysaccharides not being associated with the aggregates formed by the *exoS* strain, the cells are aggregating in an ordered fashion. This can be seen in figure 4-1A and 4-2A. All cells within an aggregate are arranged next to each other side-by-side along their longest surfaces (Fig 4-2A). These images are of cells on the coverslip suggesting that the interaction between the cells and surfaces results in the cells aligning themselves laterally along their longest surface (Fig. 4-2A). Figure 4-2B and 4-2C shows an aggregate of cells in the bulk phase. In this case the cells are also aligned laterally but are viewed from the poles of the cells. This gives the aggregate a honeycomb or speckled appearance due to the large number of cells that are arranged next to each other. A similar arrangement of cells was also seen in the parent Rm1021 when treated with succinoglycan containing-*exoS* supernatant, which results in aggregation of parent strain Rm1021 cells (Fig. 4-3A). Ordered cell arrangement also continued to occur even if the cells had been heat treated (resulting in loss of viability of the cells) (Fig. 4-3B). This, along with the loss of motility in the *exoS* mutant, suggested that the cells were not actively orientating themselves with respect to each other and some other factor must be at play.

Colloidal particles in the presence of non-adsorbing polymers will have an excluded volume around them which is entropically unfavourable for the polymers to enter. When particles come closer together the excluded volumes overlap ($V_{overlap}$), increasing the volume accessible to the diffusion of the polymers. This increases their entropy and maximises the entropy of the system. Depletion attraction will therefore be favoured by conditions in which $V_{overlap}$ is maximised. In the case of rod

shaped cells this configuration will be achieved by the cells aligning laterally (Fig. 4-4). The arrangement of the *exoS* mutant cells, with the cells stacked laterally is therefore consistent with depletion attraction. A similar interaction will also occur between the cells and the surface. In this case again it is likely that the depletion attraction would result in the cells attaching to the surface laterally which will maximise $V_{overlap}$ and the depletion interaction.

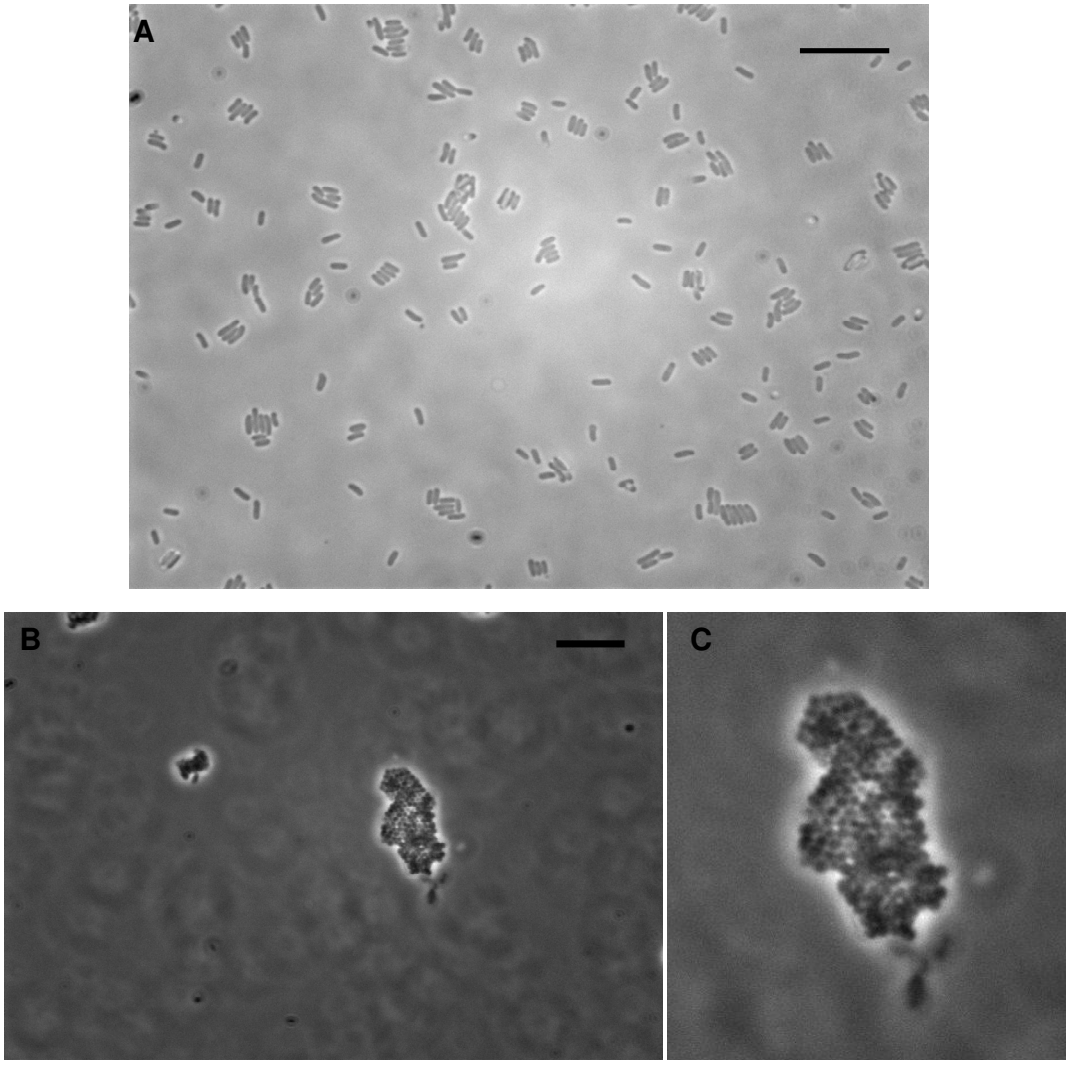


Figure 4-2. Phase contrast microscopy of the arrangement of cells in *exoS* aggregates. A. Aggregates are shown where cells are arranged laterally. B. An aggregate floating in the media where the cells are viewed from their poles rather than from their sides. As all the cells are arranged side-by-side this gives the aggregate a 'speckled' appearance. C. Digitally zoomed image of the aggregate. Bars=10µm.

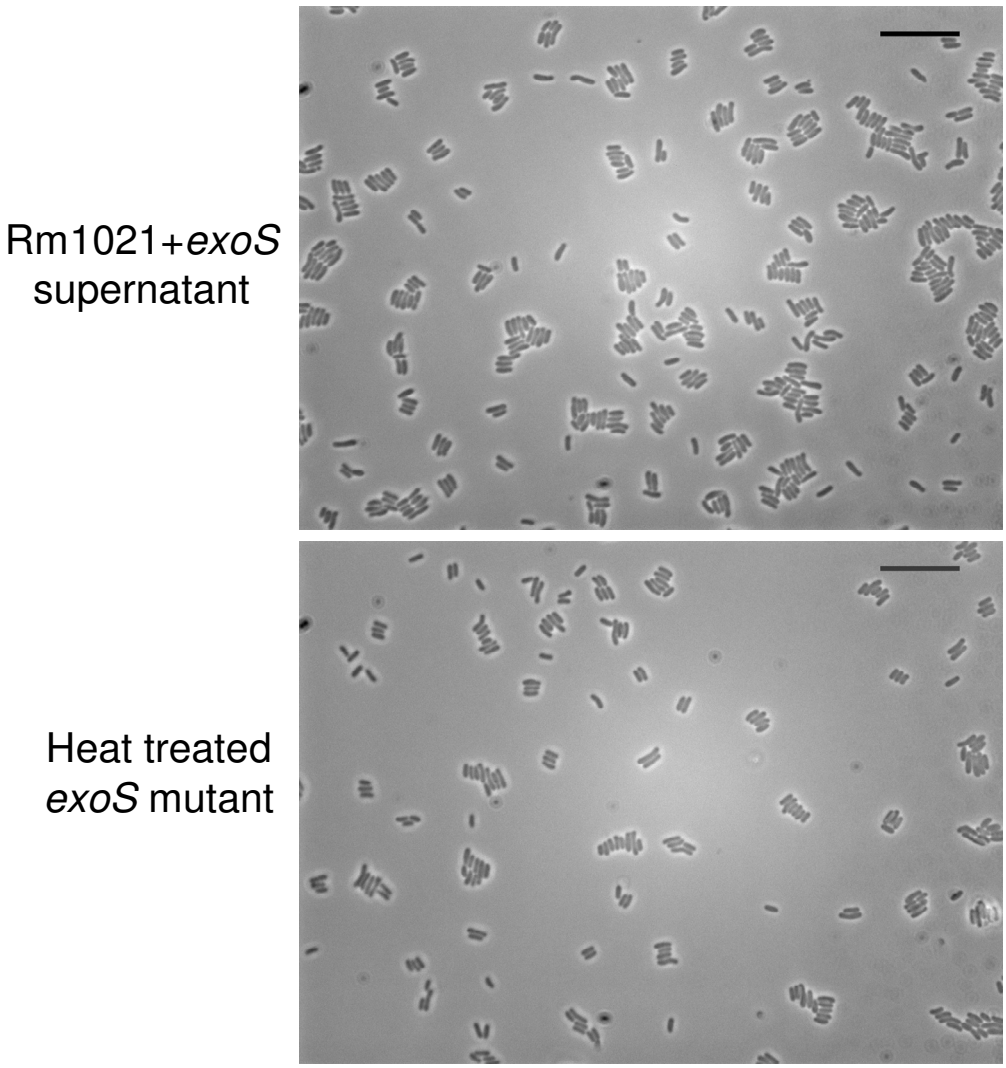


Figure 4-3 Phase contrast microscopy of aggregation. A. Rm1021 treated with supernatant from the *exoS* mutant has the same cell arrangement as seen in the *exoS* mutant. B. The *exoS* mutant treated at 65℃ for 1 hour, reducing the viability of the cells. Bars=10μm.

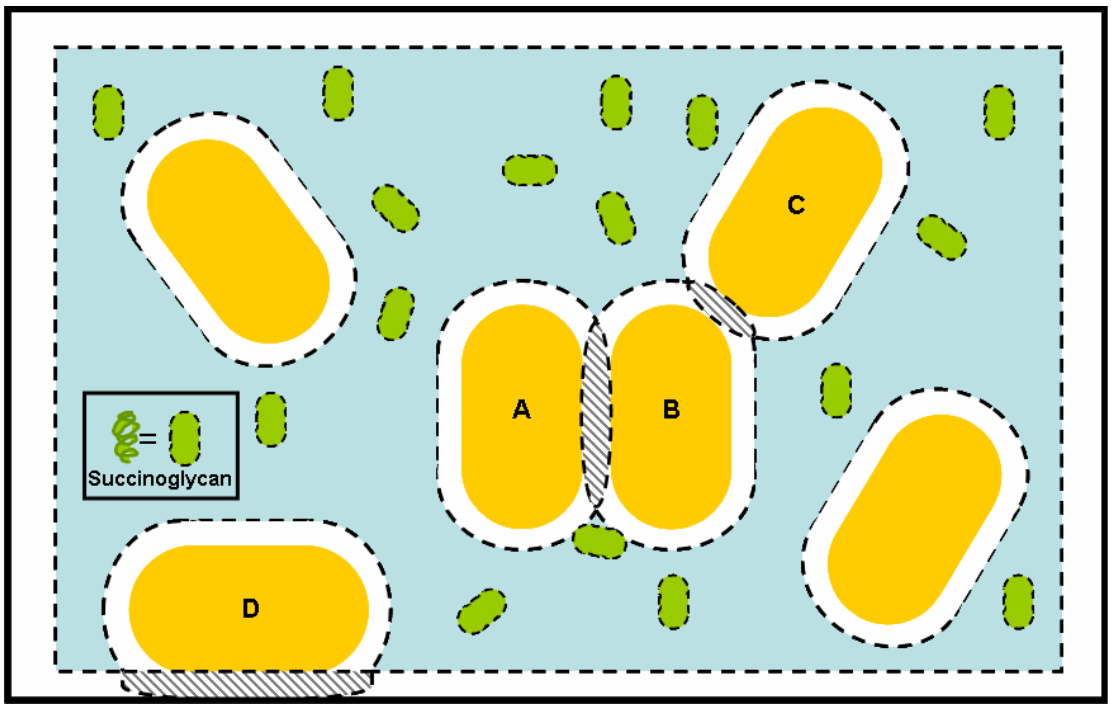


Figure 4-4. Diagram illustrating how depletion attraction influences the arrangement of cells. The reduction in entropy that would result from the succinoglycan (green) adsorbing to the surface of the cells (yellow) results in a depletion zone around the cells which is entropically unfavourable for the succinoglycan to occupy. When the cells come closer together the excluded volumes overlap ($V_{overlap}$) increasing the volume available to the diffusion of the succinoglycan, increasing its entropy. The most stable configuration for the system in which the entropy is maximised is that in which the volume inaccessible to the succinoglycan is minimised. In the case of the rod shaped bacteria lateral associations will minimise the volume inaccessible to succinoglycan by maximising $V_{overlap}$, increasing the entropy of succinoglycan and as a result, the system. The interaction between cells A and B is likely to be more stable than between B and C because $V_{overlap}$ and the depletion interaction is maximised. Similar interactions will occur between the cells and the surface as shown for cell D.

Finally two additional characteristics of depletion attraction were tested. First depletion attraction is reversible, whereas bridging flocculation is generally irreversible [107]. In the case of the *exoS* mutant the cells can be redispersed after destabilisation has occurred. The cells will then show the same pattern of sedimentation as seen previously, suggesting that the aggregation in the *exoS* mutant is reversible.

4.2 Visual observation of phase separation of *S. meliloti* with exogenous succinoglycan is consistent with depletion attraction

It therefore appeared that the aggregation by succinoglycan seen in the *exoS* mutant could indeed be attributed to depletion attraction. To investigate this hypothesis further, the relationship between polymer concentration and destabilisation of the cultures was studied. Depletion attraction is a "crowding" phenomenon and may be referred to as macromolecular crowding [105]. As such for a given polymer concentration depletion will occur more strongly at higher cell concentrations and the critical polymer concentration needed for destabilisation to occur will decrease with cell concentration. This is in contrast to bridging flocculation where the critical polymer concentration increases with cell concentration, as more polymer will be needed to bridge the cells at higher concentrations. Therefore to test the depletion attraction hypothesis succinoglycan was isolated and added exogenously to defined concentrations of cells.

4.2.1 Isolation and purification of succinoglycan

To isolate succinoglycan it was decided that the cells needed to be grown in a minimal medium as this would make it easier to isolate a purer product. LB is a complex medium which could potentially make purification of the succinoglycan more difficult. The cells were therefore grown in M9 which is a minimal medium and has been used in the characterisation of succinoglycan in previous studies [73]. The growth of the cells was measured in M9 media in the same way as LB_{MC}. The average growth rate was slower in M9 than in LB_{MC} (average growth rate= $0.15h^{-1}$ M9, $0.23h^{-1}$ LB_{MC}); the cells followed a typical growth pattern but did not reach as

high an OD_{600} as they do in LB_{MC} (Rm1021 LB_{MC} OD600: 4.39 after 39 hours/Rm1021 M9 OD600: 3 after 54 hours) (Fig. 4-5). The sedimentation of the strains was compared by transferring late exponential phase cells into cuvettes and incubating without shaking for 24 hours. In M9 the cells have the same phenotype as in LB_{MC}; the *exoS* mutant culture has destabilised within 24 hours, while the other strains remained stable, turbid dispersions, with a far slower rate of sedimentation (Fig. 4-6). In M9 the strains produce more exopolysaccharide than in LB_{MC} except strains carrying the *exoY* mutation, which do not produce any exopolysaccharide as the mutation they carry prevents them from synthesising succinoglycan (Table 4-2).

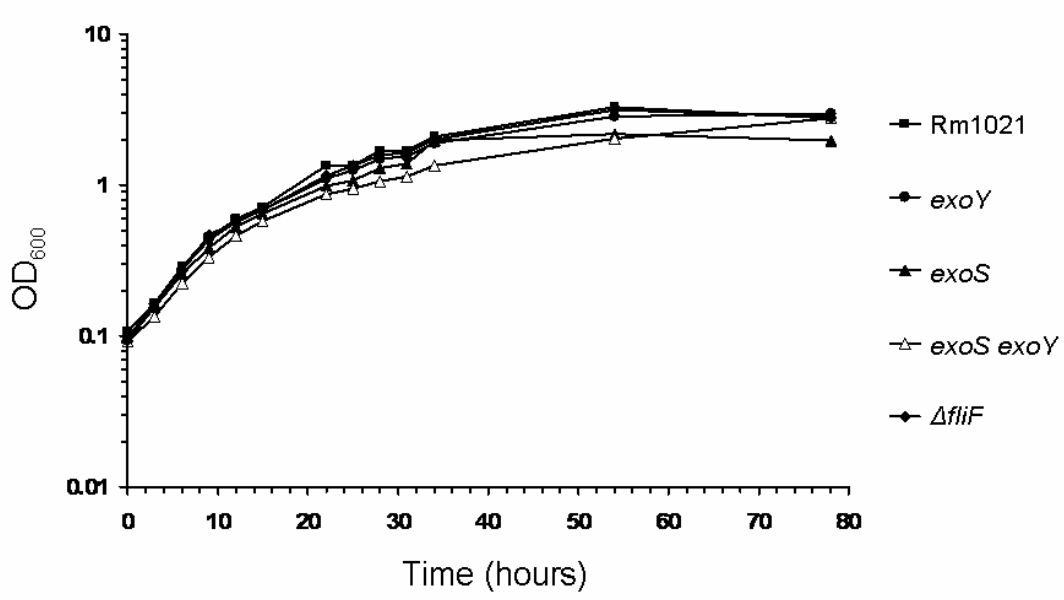


Figure 4-5. Growth curve of the *S. meliloti* Rm1021 strains in M9. Strains were grown to exponential phase in LB_{MC}, washed and inoculated into M9 before being grown at 30°C, 200rpm, with growth being monitored over the time shown. Experiments repeated with the same results.

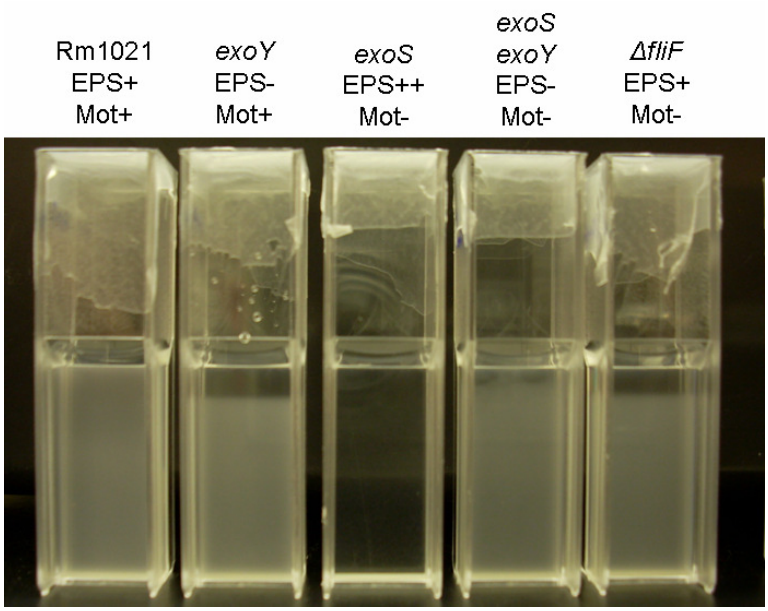


Figure 4-6. Photograph of the sedimentation of the strains after 24 hours of incubation without shaking. Cells were grown to late exponential phase in M9, transferred into cuvettes before incubation and observation. Experiments repeated with the same result.

Table 4-2. Measurement of succinoglycan by anthrone-sulphuric acid assay. Values in M9 are compared with those obtained from LB_{MC}. Polysaccharide was precipitated from the late exponential phase cultures, resolubilised and assayed. SD; standard deviation obtained from triplicates per experiment.

	Average µg glucose OD_{600}^{-1} ml^{-1} M9 (± SD)(n=3)	Average µg glucose OD_{600}^{-1} ml^{-1} LB_{MC} (± SD)(n=3)
Rm1021	8.00 (± 6.44)	0.07 (± 0.15)
exoY	-5.5 (± 3.47)	0.15 (± 0.22)
exoS	71.74 (± 28.87)	50.78 (± 16.54)
exoSexoY	-9.57 (± 1.50)	0.00 (± 0.11)
Δ*fliF*	10.27 (± 5.16)	0.31 (± 0.06)

It therefore appeared that M9 was a suitable medium for the large scale isolation and purification of succinoglycan to test the depletion attraction hypothesis. Two different protocols for the extraction of succinoglycan were tested based on established protocols for other bacterial-polysaccharide systems (Fig. 4-7) [73, 107, 116]. Due to the quantities of succinoglycan required it was decided that method two

would be used. This circumvented the need for the initial lyophilisation step of the culture supernatant, which was both time consuming and could lead to the formation of protein aggregates that contaminate the final product [107]. Analysis of the final isolated, purified succinoglycan by UV absorbance showed that there appeared to be no DNA and that it contained ~1% protein, which is similar to the purity of the product obtained by Tuinier et al. using this protocol [107]. Use of ultrafiltration rather than lyophilisation to concentrate the succinoglycan sample also meant that only the HMW succinoglycan was present in the final sample (MWCO of the filter, 300KDa). As it appeared that the mechanism of aggregation by succinoglycan was depletion attraction it seemed likely that it would the HMW form of the polymer which would be responsible for aggregation.

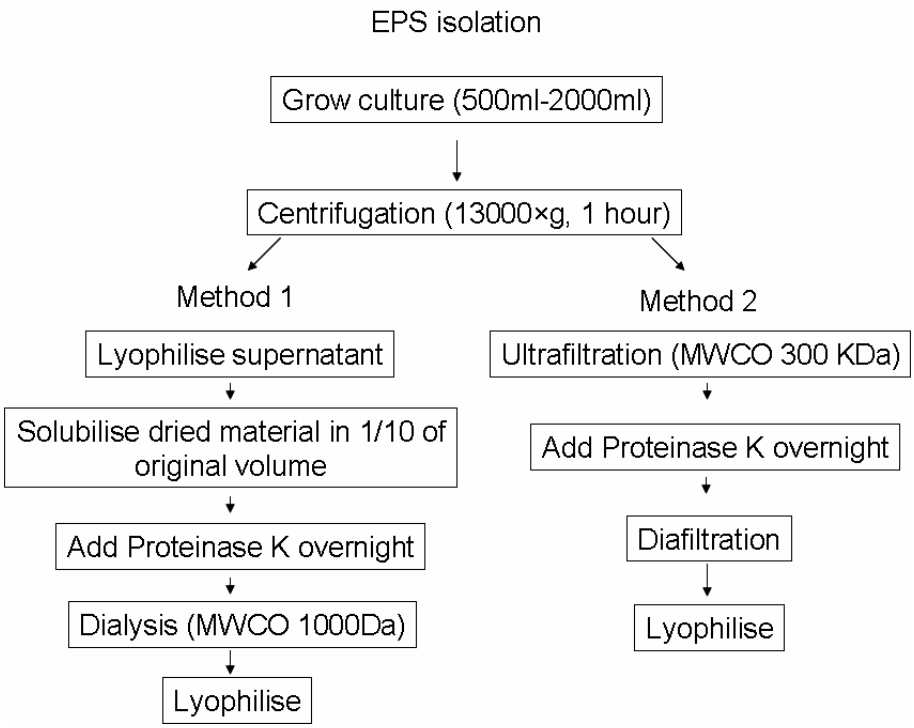


Figure 4-7. Flow diagram of the two different methods used to isolate succinoglycan from the culture supernatant of the *exoS* mutant.

4.2.2 Characterisation of succinoglycan by light scattering

The determination of molecular weight of microbial extracellular polysaccharides presents problems that are not associated with the determination of molecular weight of other macromolecules such as proteins [37]. Size exclusion chromatography (gel filtration chromatography) has been used in some studies for the determination of molecular weight of polysaccharides. However many polysaccharides are viscous which can be problematic for this type of chromatography. Further to this, determination of molecular weight relies on the use of standards, which also presents problems. Gel filtration chromatography actually measures hydrodynamic volume or the size of the molecule, which in turn is related to molecular weight [136]. For accurate determination of molecular weight the calibration standards must therefore have the same relationship between molecular size and molecular weight as the substance of interest [136]. Pullulan is now available as a standard for the determination of molecular weight of polysaccharides. Pullulan is a neutral polysaccharide consisting of maltotriose units linked by α-1,4-linkages. Each maltotriose unit is linked to another via α-1,6- linkages. Pullulan is a flexible polymer and will therefore give an overestimate of the molecular weight of a stiffer polymer [73]. Succinoglycan isolated from M9 supernatant eluted from a sepharose 4B gel size exclusion column in the same fraction as 1.5MDa pullulan standard suggesting that the HMW succinoglycan had similar molecular weight. This was also similar to the value obtained by Ridout et al. using size exclusion chromatography with pullulan standards [73]. However as pullulan and succinoglycan have very different structures, light scattering was used to gain a more direct measurement of

the size and molecular weight of succinoglycan that does not rely on the use of standards, as well as allowing some insights into the properties of the polymer.

Whenever light (a form of electromagnetic radiation) interacts with matter, scattering occurs. This is because the incident beam generates a dipole moment in a molecule, which acts as a source of electromagnetic radiation, usually referred to as scattered light. The size of the dipole moment generated and the intensity of the resulting scattered light depends on the polarizability of the molecule, which in turn is related to the molecular weight of the molecule. Measurement of the intensity of the scattered light can therefore be used to measure the molecular weight of the molecule in question.

Molecular weight is determined by measuring the intensity of the light scattered by the sample at different concentrations of the sample and from different scattering angles [45, 137]. These values can then be applied to the Rayleigh equation [45, 137]:

$$\frac{KC}{R(\theta)} = \left(\frac{1}{M} + 2A_2C\right)P(\theta)$$

where $R(\theta)$ is the Rayleigh ratio; the ratio of the intensity of scattered light from the sample to the intensity of the incident beam, M is the weight average molecular weight of the sample, A_2 is the second virial coefficient, a property that describes the interaction strength between the particles and the solvent, C is concentration of the sample, and $P(\theta)$ is the angular dependence of sample scattering intensity [45, 137].

K is an optical constant that collects into one term a number of parameters:

$$K = \frac{2\pi^2}{\lambda_0^4 N_A}\left(n_0 \frac{dn}{dc}\right)^2$$

N_A is Avogadro's constant, λ_0 laser wavelength, n_0 solvent refractive index and $\frac{dn}{dc}$ is the refractive index increment, the change in refractive index as a function of the change in concentration of the sample [45, 137]. In this study the refractive index increment was taken from the literature from Nakanishi and Norisuye (2003) [138]. The $P(\theta)$ term embodies the angular dependence of sample scattering intensity, related to the scattering generated by macromolecules such as the succinoglycan being measured here [45, 137]. When the particles are large enough, multiphoton scattering can occur. As a result there may be destructive interference of light scattered from different positions of the same particle which reduces the intensity of the scattered light [45, 137]. This problem could in theory be circumvented by measuring the scattering from zero scattering angle, as at this angle the path lengths of the scattered light would be equal and no destructive interference would occur [45, 137]. However this is not possible due to practicalities; at zero angle most of the light would be transmitted rather than scattered. As the scattered and transmitted light have the same wavelength they cannot be discriminated and the detector would be saturated. Therefore the intensity of the scattered angle is measured at a number of angles and extrapolated back to zero [45, 137].

To get a value for the molecular weight of the sample a double extrapolation method was used in a Zimm plot [45, 137]. The intensity of the light scattered from the succinoglycan sample was measured at various angles (θ) and at various concentrations of succinoglycan (0.2, 0.3, 0.4 and 0.5 g/l). These values were then

used to generate a graph of $\frac{KC}{R(\theta)}$ against $\sin^2\left(\frac{\theta}{2}\right)+kC$ where k is an arbitrary spacing factor. A double extrapolation was then carried out; extrapolation to zero angle and zero concentration. The intercept of these lines gives $\frac{1}{M_W}$ (Fig 4-8). The succinoglycan weight average molecular weight was found to be $5.63 \pm 0.56 \times 10^5$ Da. A previous study working with Rm1021 succinoglycan measured the molecular weight as 1.2×10^6Da, using chromatography with pullulan as a standard which the authors admitted was likely an overestimate for the more rigid succinoglycan [73].

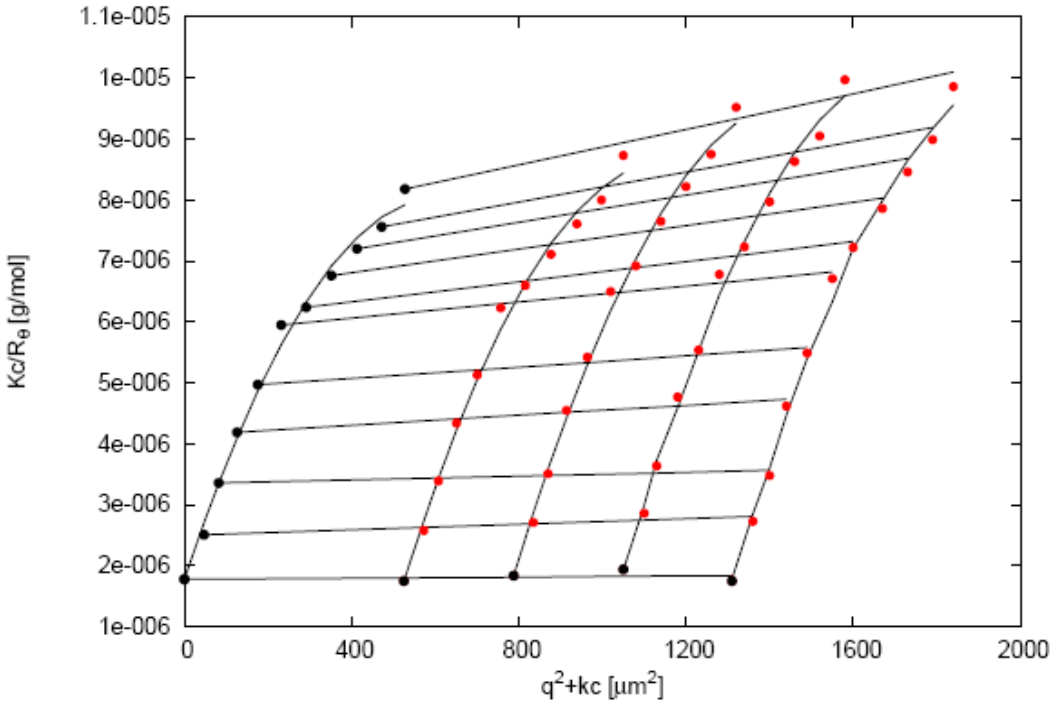


Figure 4-8. Zimm plot of static light scattering of succinoglycan isolated from supernatant of *exoS* mutant grown in M9. Red points are raw data while black points and lines represent extrapolations to zero concentration and zero scattering angle. Graph drawn by Laurence Wilson.

4.2.3 Visually observed phase diagram of S. meliloti cells in the presence of succinoglycan is consistent with depletion attraction

The isolation of succinoglycan allowed observation of the effect of altering succinoglycan concentration on the stabilisation of different concentrations of *S. meliloti* cells. The *exoY* mutant was harvested in late exponential phase and diluted to concentrations ranging from 2.5% (w/v) to 20% (w/v) in phosphate buffered saline (PBS), allowing the stability of the cultures to be easily observed. Figure 4-9A shows how the addition of 0.05% (w/v) succinoglycan results in destabilisation over the range of concentrations of *S. meliloti*, leaving the cells sedimented at the bottom of the cuvette and a clear upper phase, as previously seen in the *exoS* mutant. Without the addition of succinoglycan the cultures remain turbid, stable dispersions (Fig. 4-9A). The critical polymer concentration for destabilisation of the cultures to occur is expected to decrease with increasing cell concentration if the aggregation were occurring by the depletion attraction mechanism. The *exoY* mutant-succinoglycan mixtures were therefore diluted several times to find out at what concentrations of *S. meliloti* the succinoglycan concentration is no longer high enough to cause aggregation and destabilisation. After each dilution the cultures were scored for stability. Figure 4-9B shows the results of such dilutions. In this case at a succinoglycan concentration of 0.03% (w/v) the lower concentrations of *S. meliloti* are now stable, whereas the higher cell concentrations are still unstable, albeit with a more turbid upper phase than previously. This suggested that as the concentration of succinoglycan becomes critical it is the lower concentrations of *S. meliloti* that stabilise first.

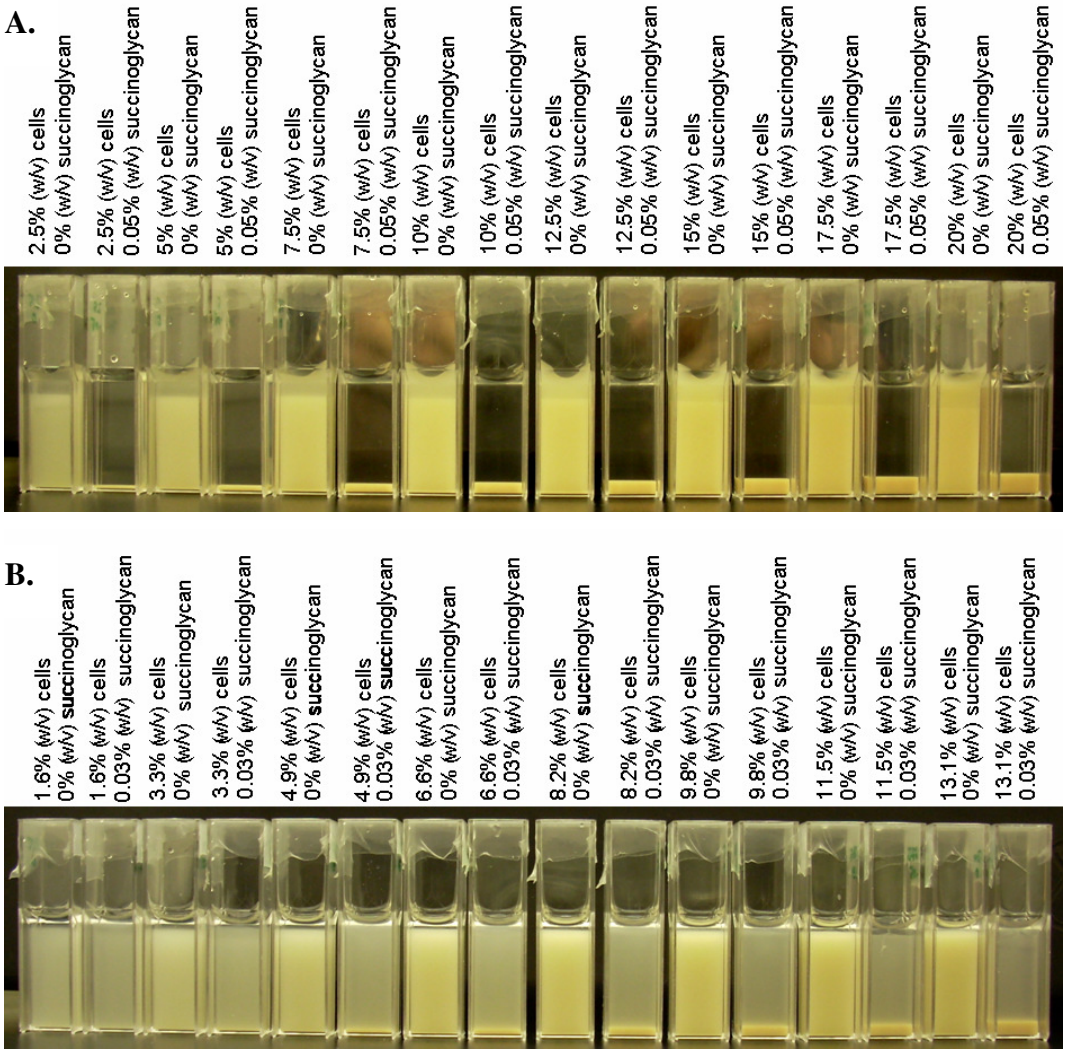


Figure 4-9 Stability of cultures in presence of isolated succinoglycan, in PBS. The *exoY* mutant was grown to late exponential phase and then diluted to a range of concentrations with the addition of succinoglycan where indicated. A. Cultures destabilise in presence of 0.05% (w/v) succinoglycan. B. Dilution of the cultures to 0.03% (w/v) succinoglycan has resulted in the lower cell concentrations now stabilising to the same extent as the controls, whereas the higher cell concentrations are still sedimenting at a higher rate than their respective controls.

The change in turbidity of the upper phase as the dispersions approach stability following dilution can also be monitored by measuring the change in OD_{600} (Fig. 4-10). This supports the visual observation of the mixtures. The OD_{600} increases with each dilution, until a plateau is reached signifying stability (Fig. 4-10). The upper phase of the mixtures with an initially lower concentration of cells increase in

turbidity and reach stability faster than the dispersions with higher cell concentrations (Fig 4-10).

The relationship between stability and concentration of the cell-polysaccharide dispersions can also be represented in the form of a phase diagram (Fig. 4-11). Each point on the diagram represents a cuvette which has been scored for stability after 24 hours of static incubation (Fig. 4-11). This data again shows that the lower concentrations of cells reach stability after fewer dilutions than the higher concentrations of cells (Fig. 4-11). A line drawn showing the boundary for the transition from an unstable to a stable state illustrates that the polymer concentration required to induce instability decreases with increasing cell concentration (negative slope) (Fig. 4-11). This is consistent with the hypothesis that depletion attraction is the underlying mechanism for succinoglycan induced aggregation in *S. meliloti*.

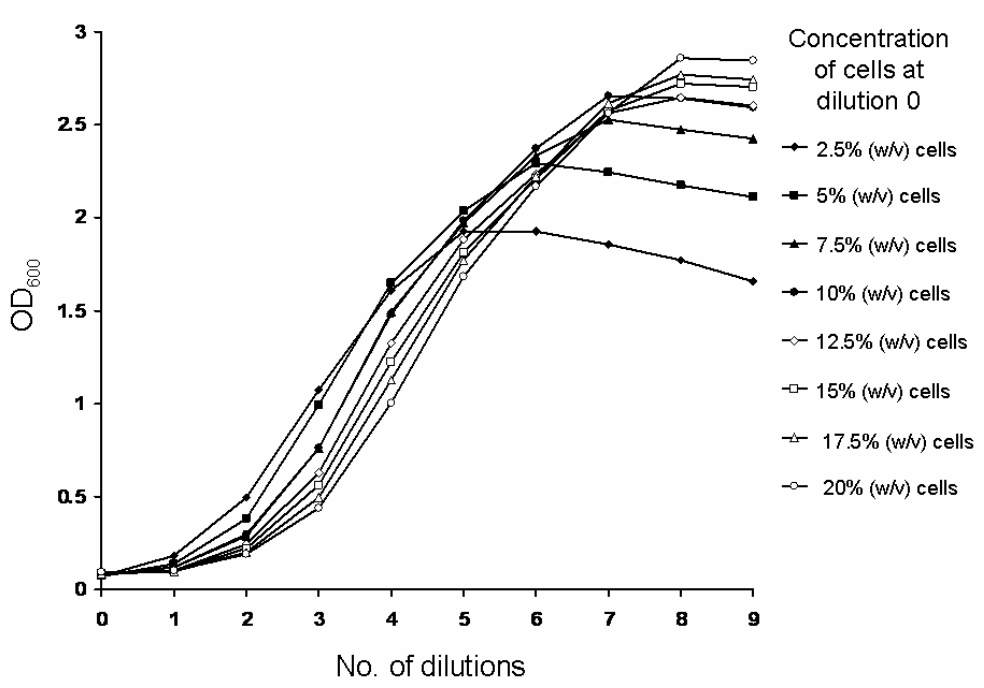


Figure 4-10. OD_{600} of the upper phase of the cultures after 24 hours of static incubation, over successive dilutions. Cells were dispersed in PBS and succinoglycan was added at an initial concentration of 0.05% (w/v). Experiments were performed with triplicates for each sample and repeated at least once.

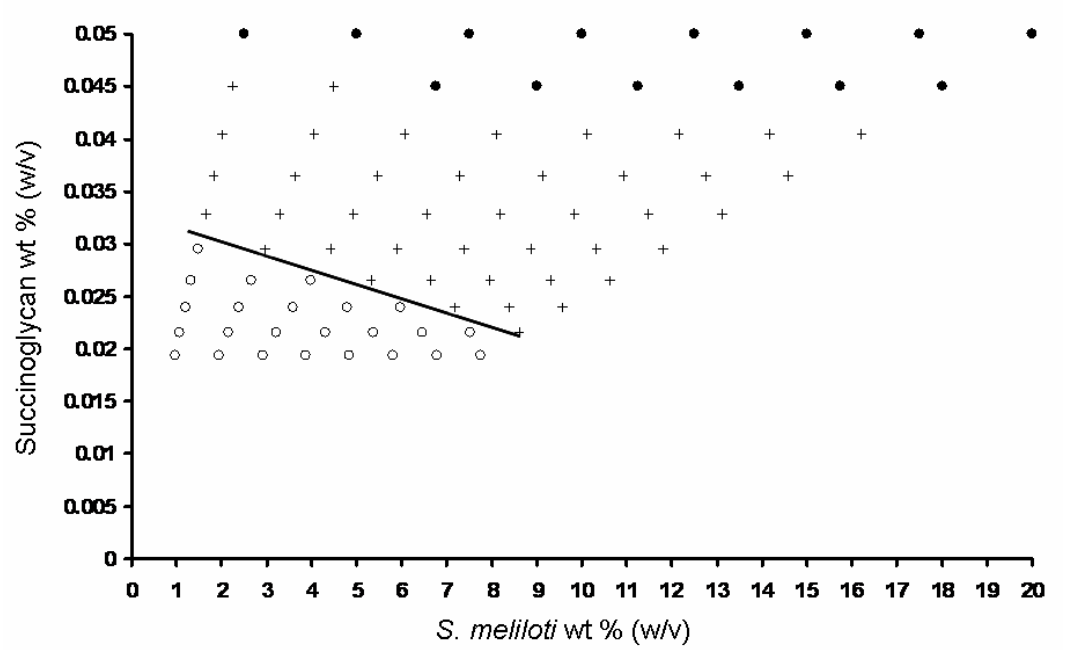


Figure 4-11. Phase diagram of various concentrations of *S. meliloti*- succinoglycan cultures in PBS. Filled circles are unstable cultures, + are unstable cultures with a turbid upper phase and open circles are stable cultures. The line is drawn as a guide to the eye and shows the critical polymer concentration at which the cultures go from unstable to stable cultures. Experiments were performed with triplicates of each sample and were repeated at least once.

4.2.4 Succinoglycan depletes more strongly in deionised water than in PBS

The above experiments were repeated in sterilised deionised water. It is highly unlikely *S. meliloti* would ever be exposed to pure water in its natural environment. However experiments testing depletion attraction in bacteria with synthetic polymers have been carried out in water and so this provided a comparison with those earlier studies [117, 118].

As above, cells were harvested in late exponential phase and redispersed in water before being diluted to the required concentration along with the addition of succinoglycan. The *exoY* mutant was again used to ensure that the cells were not capable of producing succinoglycan. Destabilisation of the cultures was both visually observed and the OD_{600} of the upper phase was measured. Cultures were allowed to settle for 24 hours before measuring, and then subsequently remixed and diluted.

As with the PBS treatments, addition of succinoglycan results in destabilisation of the cultures (Fig. 4-12). Figure 4-13 shows the OD_{600} change of the upper phase as a factor of the subsequent dilutions. There is a far longer lag than that observed when the cells and succinoglycan were in PBS (Fig. 4-13 and 4-10). This again shows that the lower concentration of cells reach a plateau before the higher concentration of cells (Fig. 4-13). Coupled with the visual observation, the lower cell concentrations become stable before the higher cell concentrations. Figure 4-14 is the phase diagram for the *exoY* mutant-succinoglycan mixtures in water. This shows that depletion continues to occur at far lower concentrations of polymer than in PBS. It should also be noted that a line showing the transition form unstable to stable has a

downward trend. The critical polymer concentration therefore again decreases with increasing cell concentration, consistent with depletion attraction (Fig. 4-14).

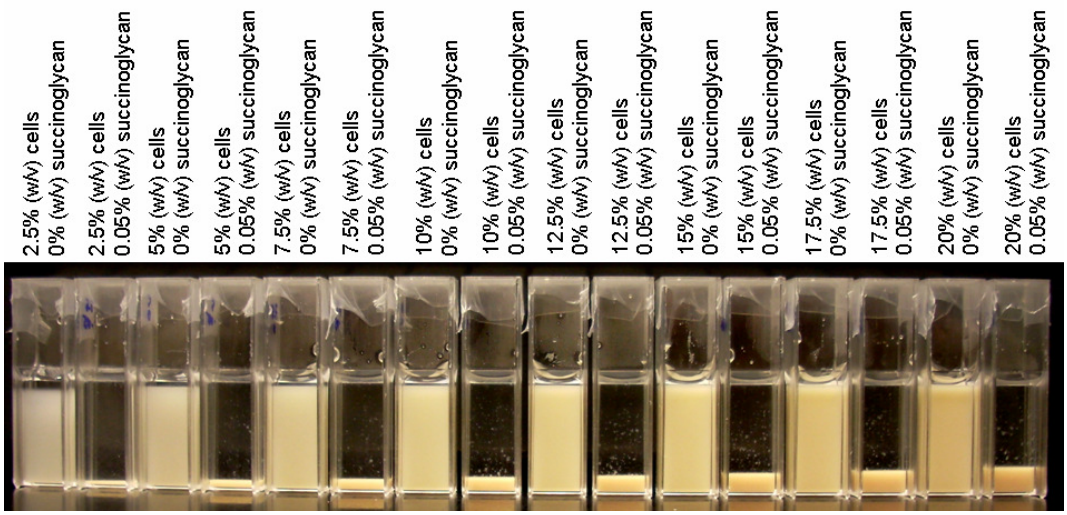


Figure 4-12. Addition of succinoglycan to *S. meliloti* Rm1021 *exoY* mutant cells dispersed in water results in destabilisation of the cultures as seen in PBS.

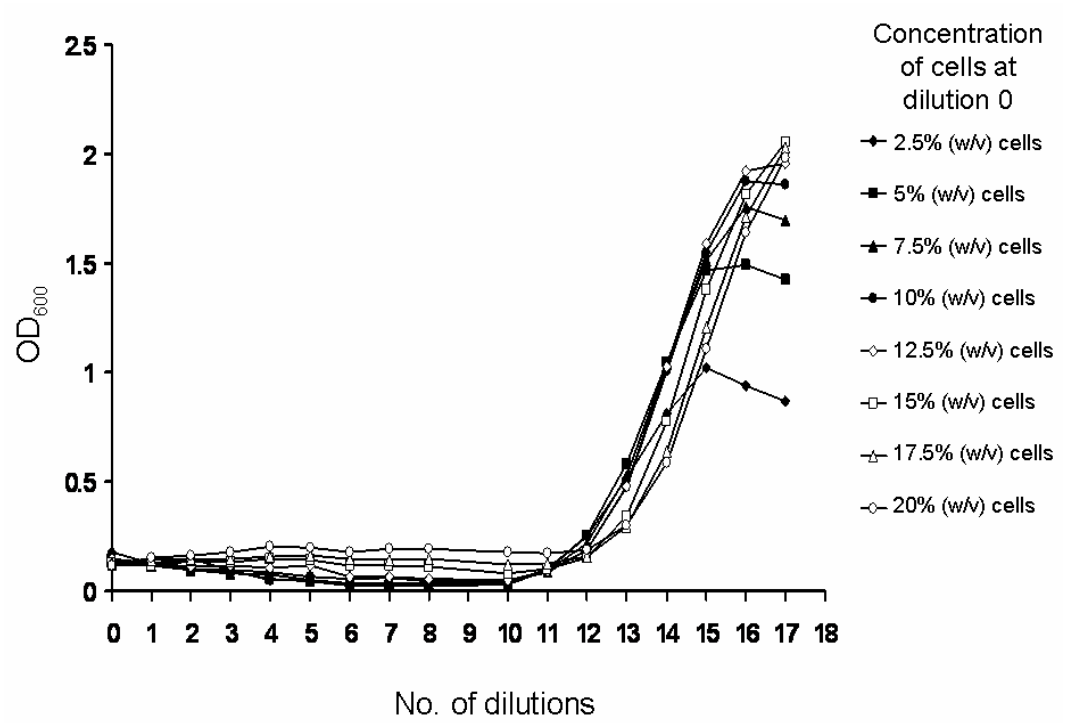


Figure 4-13. Cells were dispersed in water at the concentration showed in the figure legend with the addition of 0.05% succinoglycan (w/v). OD_{600} of the upper phase was then monitored over successive dilutions. Cultures were incubated without shaking for 24 hours before OD_{600} was measured.

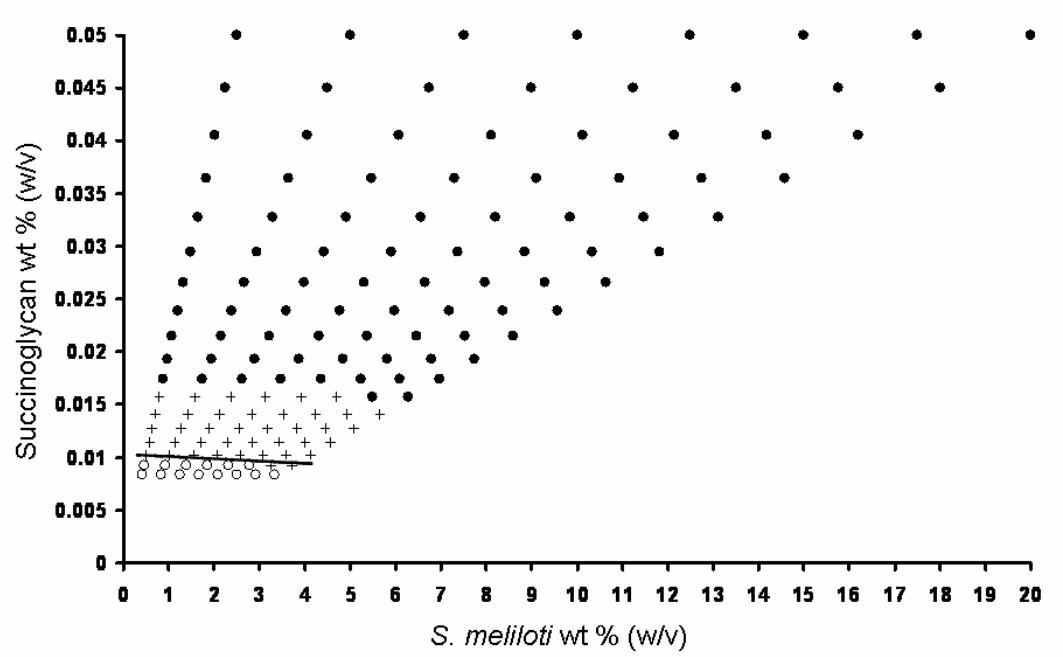


Figure 4-14. Phase diagram of *S. meliloti*- succinoglycan cultures in water. Line represents the critical polymer concentration for the transition between unstable and stable cultures. Filled circles are unstable, + are unstable with a turbid upper, open circles represent stable cultures.

4.2.4 Growth of the parent strain Rm1021 in minimal media results in unstable cultures

The *exoS* mutant, when grown in LB_{MC} and M9 had been shown to form aggregates, which result in destabilisation of the cultures when they are incubated without shaking subsequent to growth. This is due to the overproduction of succinoglycan and resulting depletion attraction of the cells.

The parent strain Rm1021 does not display any aggregative behaviour in either LB_{MC} or M9. This may be due to the fact that the parent strain does not produce large enough quantities of succinoglycan to induce aggregation. To test whether the parent was capable of aggregation the strain was grown in M9 without

the addition of NH_4Cl but with a good carbon source. This has been shown to increase succinoglycan biosynthesis in *S. meliloti* Rm1021 [86] and exopolysaccharide biosynthesis in bacteria in general [37].

Due to the limited growth in this media, the bacteria were first grown in LB_{MC} and then sub-cultured into the minimal M9 at an OD_{600} of 0.5, to ensure a large enough bacterial population for the experiments. A growth curve is shown in Fig. 4-15 and shows the limited growth of the strains in this media. Succinoglycan biosynthesis of the strains in the minimal M9 was measured using the anthrone-sulphuric acid assay and compared to succinoglycan production in LB_{MC} (Table 4-3). This showed that under these conditions the parent strain Rm1021 produces far more succinoglycan than in LB_{MC}.

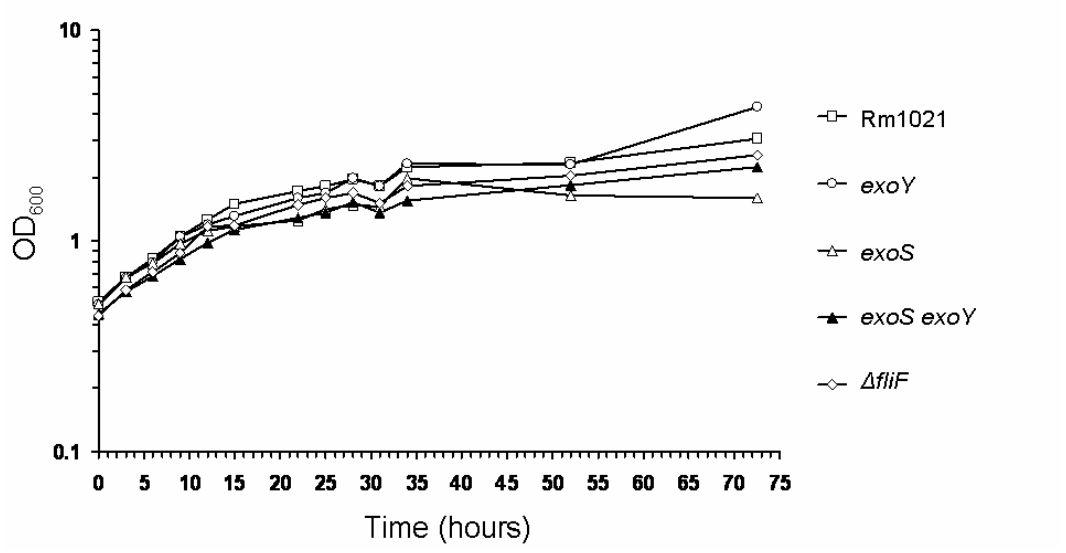


Figure 4-15. Growth curve of *S. meliloti* Rm1021 strains in minimal M9 (M9 without NH4Cl). Strains were grown to exponential phase in LB_{MC}, washed, inoculated into the minimal M9 at a starting OD_{600} of 0.5 and grown at 30°C at 200 rpm. OD_{600} was monitored at the time points indicated. Experiments repeated with similar results.

Table 4-3. Succinoglycan biosynthesis produced by the strains in M9-NH_4Cl and compared to synthesis in LB_{MC} as measured by the anthrone sulphuric acid assay. Experiments were performed with triplicate samples and repeated at least once.

Strain	Average µg glucose equivalents OD_{600}^{-1} ml^{-1} ± SD in M9-NH4Cl	Average µg glucose equivalents OD_{600}^{-1} ml^{-1} ± SD in LB_{MC}
Rm1021	35.83 ± 5.84	0.07 ± 0.15
exoY	0.24 ± 1.04	0.15 ± 0.22
exoS	139.69 ± 18.38	50.78 ± 16.54
exoSexoY	0.84 ± 1.03	0.00 ± 0.11
Δ*fliF*	40.50 ± 3.72	0.31 ± 0.06

The strains were grown for 24h and then transferred into cuvettes for observation of stability. Figure 4-16 shows that in contrast to LB_{MC} the parent strain Rm1021 and the Rm1021 Δ*fliF* mutant, both capable of producing succinoglycan, had sedimented and were thus unstable. Strains carrying the *exoY* mutation however remained stable after static incubation, sedimenting at a far slower rate than the parent or the Δ*fliF* mutant. Interestingly the *exoS* mutant, when grown in the M9 minimal medium has lost the unstable culture phenotype. The mutant showed no sedimentation at all, even after several days and appeared to be completely stable.

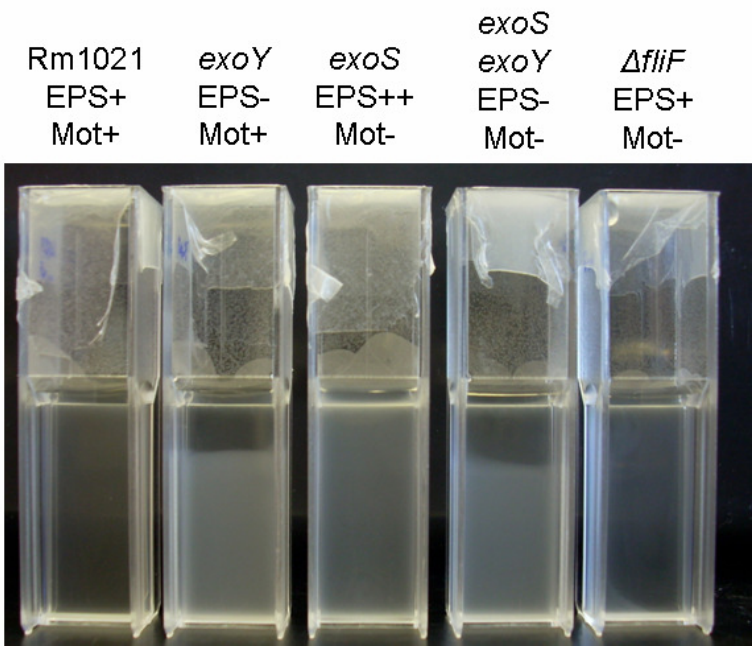


Figure 4-16. Sedimentation of the *S. meliloti* Rm1021 strains after 24 hours incubation without shaking. Cultures were grown in minimal M9 (M9 without NH_4Cl) for 24 hours at 30℃ and 200rpm, before 1ml was inoculated into cuvettes. Experiments were repeated and gave the same observation.

4.3 Discussion

The aim of this chapter was to understand the mechanism underlying the enhanced aggregation and resulting destabilisation of cultures of the *exoS* mutant by succinoglycan. It has been shown that the physicochemical mechanism underlying aggregation is depletion attraction otherwise known as macromolecular crowding. This is the first time that an exopolysaccharide being secreted by bacteria has been shown to result in aggregation of those bacteria via depletion attraction.

4.3.1 S. meliloti aggregates lack an extracellular matrix and arrangement of the cells is indicative of depletion attraction

In many bacterial systems, aggregation is thought to be due to bridging flocculation, whereby the polymer (usually polysaccharide) adsorbs to the surfaces of the cells sticking them together into aggregates (flocs) and biofilms. The depletion attraction hypothesis relies on the polymer, in this case polysaccharide, not adsorbing to the surface of the cells. The fact that this may be the case for *S. meliloti* aggregation by succinoglycan was suggested by the net relative charge of the cell surface and the polymer. Electrophoretic mobility of the *S. meliloti* cells showed that all the strains were carrying an overall negative charge. Succinoglycan carries acidic substituents that also carry a negative charge at physiological pH, suggesting that electrostatic repulsion would inhibit the polysaccharide and the cells attaching to each other. The attachment of the succinoglycan would also lead to a decrease in entropy of the polysaccharide which is unfavourable. Therefore the adsorption of succinoglycan to the surface of *S. meliloti* is unlikely due to both electrostatics and reduction in entropy.

Support for the bridging flocculation hypothesis for bacterial aggregation appears to be mainly based on visual observation of aggregation of bacteria producing polysaccharides. TEM and SEM images often show fibrillar or dense material around aggregates (although the preparative conditions often requires dehydration and can lead to aberrations in the images), phase contrast microscopy has been used to visualise the matrix surrounding the cells and the use of fluorescent lectins in confocal microscopy has also been used to show polysaccharides encasing bacterial aggregates and biofilms [19, 25, 38, 53, 139]. In the case of *S. meliloti* however, the

aggregates formed by the *exoS* mutant were striking in that there is no polysaccharide matrix surrounding the cells. This was shown both by phase contrast microscopy and also fluorescence microscopy, which was used to visualise succinoglycan by using the fluorescent dye calcofluor which binds to succinoglycan. Instead, succinoglycan is dispersed throughout the medium and does not appear to adsorb to the cells. This provides key support for the hypothesis that the mechanism of aggregation is depletion attraction; the non-adsorbing succinoglycan will result in the imbalance in osmotic pressure when the cells come closer together which drives the attraction between particles. Further evidence was provided by the organised arrangement of the cells. The *exoS* mutant or cells treated with succinoglycan align laterally, both with regards to cell-cell and cell-surface interactions, which would maximise the depletion attraction and lead to the most stable aggregative arrangement.

4.3.2 Destabilisation of S. meliloti cultures by succinoglycan is a crowding phenomenon

S. meliloti cell suspensions in the presence of purified succinoglycan were found to be unstable; aggregation induced by the polysaccharide results in enhanced sedimentation of the cells. The methodology used to isolate purified succinoglycan meant that the HMW form of the polymer was isolated preferentially over the LMW. Light scattering measurements showed the polymer to have a molecular weight of $5.63 \pm 0.56 \times 10^5$ Da. A previous study working with Rm1021 grown in M9, measured the molecular weight of succinoglycan as 1.2×10^6Da, using chromatography with pullulan as a standard which the authors admitted was likely an overestimate for the more rigid succinoglycan [73]. Addition of this purified succinoglycan resulted in destabilisation of the cultures showing, as expected, that it

is the HMW form that is responsible for aggregation, rather than the LMW form which has a role in the initiation of the symbiosis with the plant host [123]. Addition of succinoglycan to *S. meliloti* cells showed that the critical concentration of succinoglycan required to cause destabilisation of the cultures decreased with increasing cell concentration, i.e. the aggregation effect of succinoglycan occurred more readily in crowded dispersions. This is consistent with depletion attraction and the opposite of what would be expected with bridging flocculation. This effect occurred in PBS and also water, although in water a lower concentration of succinoglycan was required for depletion attraction and destabilisation to occur (~0.03-0.02% (w/v) in PBS compared to ~0.01% (w/v) in deionised water).

The differing results in water and PBS may be due to the conformation of succinoglycan which also provides further support for depletion attraction as the mechanism driving the aggregation of *S. meliloti*. Previous studies suggests that succinoglycan is a rod-like polymer [73, 138, 140]. Rods exhibit a stronger depletion attraction than equivalent spheres [141-143]. This is due to their high aspect ratio; rods have greater rotational degrees of freedom than equivalent spheres which will lead to a greater increase in entropy when the colloidal particles overlap and increase the volume available to the rods [141-143]. In water succinoglycan depletes far more strongly- a far lower concentration (less than 0.01% w/v in water compared to 0.02%-0.03% w/v in PBS) is required to induce destabilisation of the cultures. The enhanced depletion by the succinoglycan is most likely due to the ionic strength of the media affecting the conformation of succinoglycan. It has been shown that in higher ionic strength buffers the ordered form of the polymer is favoured and that removal of the charged substituents results in stiffening of the polymer [73]. It may be

expected therefore that in water succinoglycan will be more flexible than in PBS, as in PBS the screening of the charges will favour a stiffer conformation. In measurements of the depletion attraction between colloids dispersed in rods it was found that flexible rods can increase the strength of the depletion interaction [143]. The undulations of flexible rods mean greater degrees of freedom than stiff rods, which will impact on the entropy of the system and increase the depletion interaction [143].

It is highly unlikely *S. meliloti* would be exposed to pure water in its natural environment. However earlier studies of bacterial aggregation in the presence of PSS used deionised water as the medium [117]. In these studies it appeared that an increasing concentration of PSS was required to destabilise cultures of *E. coli* as the concentration of *E. coli* increased. This is in contrast to what would be expected with depletion attraction. While the authors of this paper suggested that depletion attraction was occurring [117], it seems possible that the coiled PSS is able to take on 'loopy' configurations and make contact with positive charges present on the membrane of the bacteria leading to bridging [148].

4.3.3 Increased production of succinoglycan by the parent strain Rm1021 results in aggregation

While it had therefore been shown that the *S. meliloti* Rm1021 *exoS* mutant was capable of aggregation and that the mechanism was depletion attraction, it was still unclear whether the parent strain Rm1021 was capable of similar aggregation. It has been shown in a number of bacterial species that growth in media where an essential nutrient, such as phosphate or nitrogen is limited, but where carbon is in good supply results in the bacteria upregulating the synthesis of extracellular

polysaccharides. This appears to be a way for the bacteria to utilise the carbon source when growth is impossible due to the limitation of another nutrient. *S. meliloti* Rm1021 strains were therefore grown in M9 with a good carbon source but with no nitrogen (NH_4Cl was omitted from the media). In this media the parent strain Rm1021 produces far more succinoglycan. As a result static incubation of the culture after growth resulted in an unstable culture in the same way as had been seen for the *exoS* mutant in LB_{MC}. Strains carrying the *exoY* mutation however did not have enhanced sedimentation in this media, showing that succinoglycan was responsible for the destabilisation of the cultures. Interestingly however the *exoS* mutant when grown in the M9 lacking nitrogen was now completely stable. There was no sedimentation at all, even after several days. This appears to be due to the viscosity of the media. While the *exoS* mutant has been shown to be a constitutive over producer of succinoglycan compared to the parent strain in all media, it can still modulate levels of succinoglycan biosynthesis in response to alterations in environmental conditions [86]. It therefore appears that the upregulation of the synthesis of succinoglycan in the nitrogen-limited M9 may have resulted in an increase in viscosity of the media which stabilises the dispersion. While this result may appear contradictory to the role of succinoglycan in aggregation as outlined above, this is similar to results obtained with xanthan. Both xanthan and succinoglycan share some properties and both have uses as thickeners as food additives [37, 42]. Xanthan in particular is added to stabilise salad dressings, where it prevents destabilisation of the oil-water emulsion [114, 115]. However it has been found that xanthan in low concentrations can actually destabilise the emulsion, due to depletion attraction of the oil droplets [115]. It therefore appears that a similar

phenomenon is occurring in the *exoS* mutant culture; the overproduction of succinoglycan has now increased to a level whereby the polysaccharide stabilises rather than destabilises by depletion attraction.

For the first time it has therefore been demonstrated that succinoglycan drives aggregation of *S. meliloti* Rm1021 by depletion attraction. Given the polyanionic nature of many bacterial extracellular polysaccharides [37, 53], coupled with the entropy considerations, it is perhaps surprising that depletion attraction hasn't been considered more as a possible mechanism of bacterial aggregation. It is often unclear how extracellular polysaccharides are associated with the cell membrane. In the case of capsules there may be a covalent linker, usually in the form of a phosphodiester bond between the polysaccharide and phospholipid or lipid A in the membrane [36]. Capsule assembly at the outer surface of the cell may require specific proteins. For example WzI is responsible for assembly of the group I capsular polysaccharide produced by *E. coli*, on the outer surface of the cell; without it the capsular polysaccharide is secreted into the medium [144]. However capsular polysaccharides can be sheared from the surface of the cell due to the stability of the phosphodiester linkage between the polysaccharide and phospholipid membrane anchor [36]. Alternatively the capsule may lack a covalent anchor altogether [145]. The distinction between an exopolysaccharide and a capsular polysaccharide can therefore be ambiguous. For example in *P. aeruginosa*, where aggregates of cells have been observed in the mucoid state encased in a dense extracellular matrix, the alginate responsible for the mucoidy and increased aggregation and biofilm formation is often referred to as "capsule-like" [48, 49]. In the absence of a covalent linker there may be

several types of interaction which may facilitate adsorption of the polysaccharide and hence aggregation of the cells. Hydrophobicity can be important- an increase in the hydrophobicity of cells correlates to an increase in attachment in wastewater flocs, with hydrophobic portions of some extracellular polysaccharides providing focal points for attachment and aggregation of the cells [53]. It has been shown in some oral biofilms that successive colonisation of the biofilm is due to production of lectins by the bacteria allowing attachment to the polysaccharide laid by bacteria that have already colonised the surface [53]. Alternatively non-covalent "weak" interactions, such as Van der Waals, electrostatic forces (which as highlighted above can be repulsive as well as attractive) and hydrogen bonding may all have a role in biofilm polysaccharide-cell attachment and integrity of the biofilm or aggregate [146]. Due to the large number of functional groups present on the polymer these weak interactions can cumulatively contribute a high binding energy for the polysaccharide to the cell surface [146]. An interesting example is provided by *Staphylococcus epidermidis*. *S. epidermidis* are Gram positive bacteria that are prevalent in hospital-acquired infections; often infections result after the insertion of indwelling devices such as prosthetic heart valves [147]. In these cases the virulence is correlated to the bacteria adopting a 'biofilm lifestyle' [147]. To form biofilms the bacteria produce an extracellular polysaccharide called polysaccharide intercellular adhesion (PIA), a homopolymer of poly-N-acetylglucosamine [147]. In order to fulfil its role as an intercellular adhesion molecule, the polysaccharide is deacetylated, exposing the amino group which becomes protonated and results in a positive charge on the polymer at neutral and acidic pH [147]. It is this modification and alteration of the polymer to a polycation that the authors hypothesised was essential for it to be able

to attach to cells and cause biofilm formation [147]. It is interesting that in this case for the polysaccharide to attach and cause aggregation it appears essential that the molecule takes on a polycationic character, when so many extracellular polysaccharides are either neutral or polyanionic yet are still expected to adsorb to cells. It is clear however that the attachment of polysaccharides to the cell surface is a poorly understood phenomenon and that under the right conditions depletion attraction may be more prevalent than currently appreciated.

Chapter 5

Depletion attraction in other systems

The previous chapter demonstrated that the presence of succinoglycan in large enough amounts results in aggregation and subsequently destabilisation of *S. meliloti* cultures by depletion attraction. To find out if this effect of succinoglycan was unique to *S. meliloti* the same experiments as outlined in the previous chapter were repeated using *Escherichia coli* MG1655 and polystyrene colloids. To investigate whether depletion attraction of *S. meliloti* cells was also unique to succinoglycan, alternative polymers were tested for their effects on the stability of *S. meliloti* cultures. Xanthan is a bacterial extracellular polysaccharide produced by the plant pathogen *Xanthomonas campestris*. Polystyrene sodium sulfonate (PSS) has been used in previous studies on bacterial aggregation by depletion attraction, so this polymer was also tested on *S. meliloti* dispersions [117, 148].

5.1 Addition of succinoglycan to *E. coli* MG1655 can result in depletion attraction but is inhibited if the cells have flagella

E. coli MG1655 is a model organism that is a derivative of *E. coli* K-12 and has been maintained in the laboratory with the minimum of genetic manipulation [149]. Growth of MG1655 was characterised by generation of a growth curve, monitoring change in OD_{600} over time (Fig. 4-17). *E. coli* MG1655 grows faster than *S. meliloti* Rm1021 (Rm1021growth rate= 0.17h-1, Fig. 3-3; MG1655 growth rate= $1.2h^{-1}$, Fig.5-1).

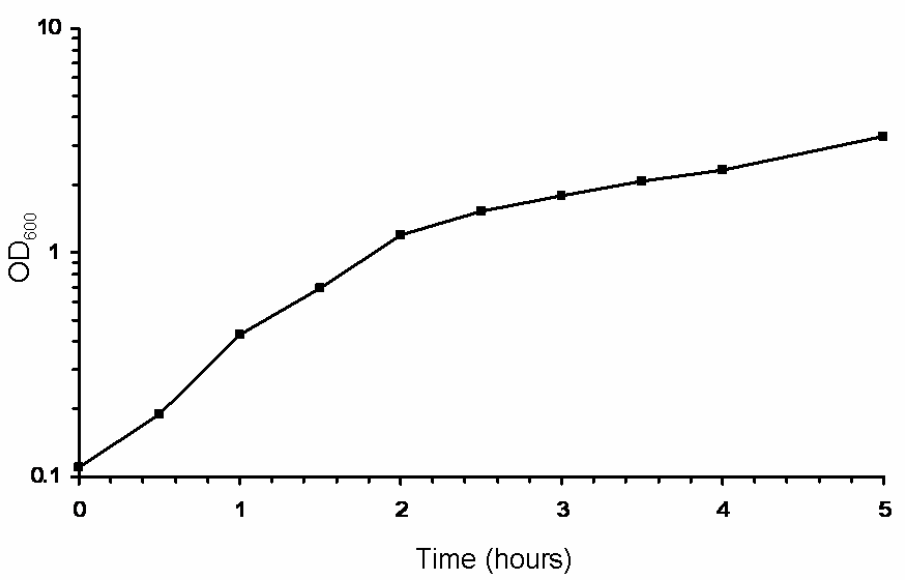


Figure 5-1. Growth curve of *E. coli* MG1655 in LB at 37°C, shaking at 200rpm. OD_{600} of the culture was monitored over the time points indicated.

As for the *S. meliloti* experiments, succinoglycan was added to *E. coli* harvested in late exponential phase with the cells diluted to concentrations ranging from 2.5% to 20% (w/v) in PBS (Fig. 5-2). As for *S. meliloti*, cells without the addition of succinoglycan remained as stable, turbid dispersions (Fig. 5-2). However while the addition of succinoglycan did appear to have some effect on the sedimentation rate of *E. coli* the polysaccharide did not result in a loss of stability as seen for *S. meliloti* (Fig. 5-2). Eboigbodin *et al.* had found that growth phase affected the kinetics of aggregation and amount of polymer required for aggregation and destabilisation of the cultures [117]. To see if growth phase was having an effect on stability of *E. coli* in the presence of succinoglycan, cells were harvested from stationary phase and incubated in the presence of succinoglycan in PBS (Fig. 5-2). In contrast to the exponential phase cultures *E. coli* MG1655 in stationary phase destabilised in the presence of succinoglycan. The controls without succinoglycan remained as stable, turbid cultures. As before the cultures were diluted to find the

critical cell-polysaccharide concentrations for destabilisation (Fig 5-3). As found in *S. meliloti* the critical succinoglycan concentration decreases with increasing cell concentration (Fig. 5-3). In stationary phase cultures of *E. coli* MG1655 at least, it therefore appears that succinoglycan can induce aggregation and destabilisation of the cultures via depletion attraction.

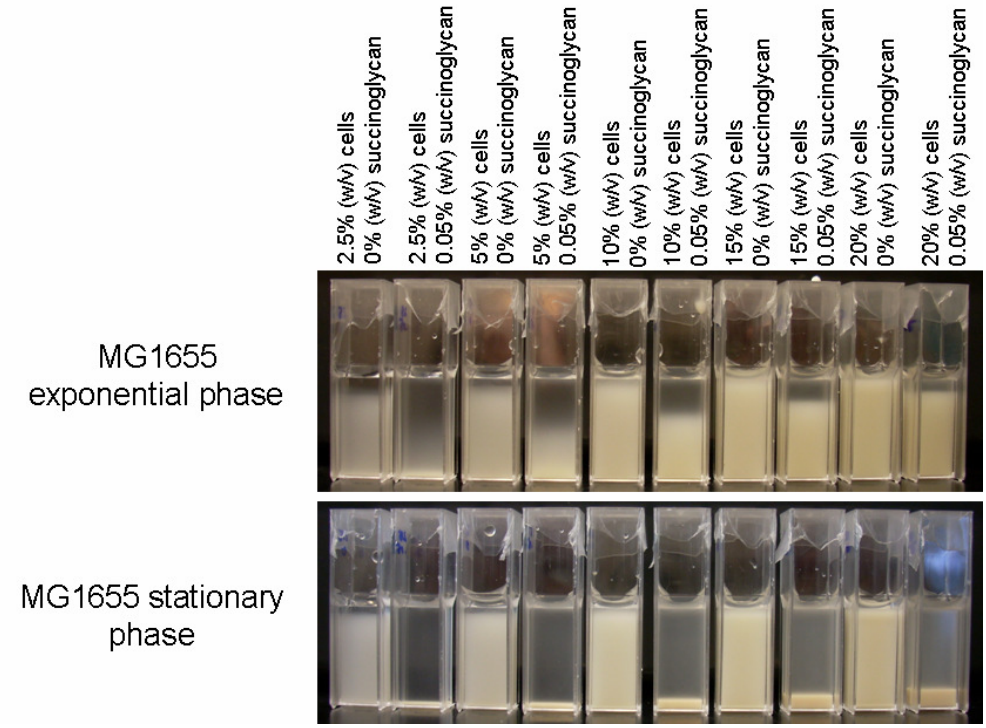


Figure 5-2 Stability of *E. coli* MG1655 cultures in the presence of succinoglycan. Cells were grown to required growth phase and diluted in PBS to the indicated concentration. Succinoglycan was added at indicated concentration. Cultures with 0% succinoglycan are controls. Experiments repeated with the same observation.

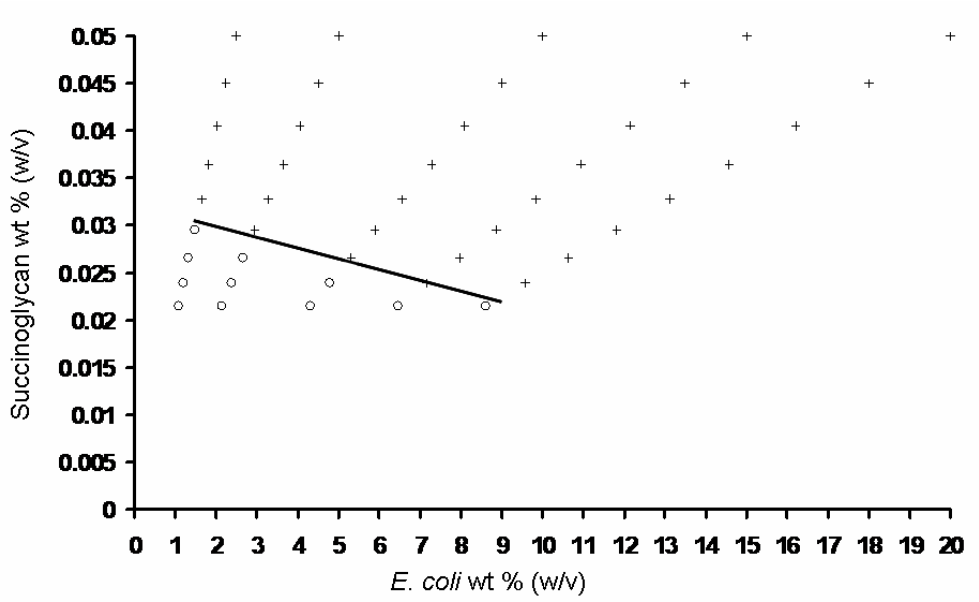


Figure 5-3. Phase diagram showing stability of various concentrations of stationary phase E. coli MG1655 to the presence of succinoglycan. + represent unstable cultures with a turbid upper. Open circles are stable cultures. Line drawn as guide to the eye, represents the transition from unstable to stable cultures.

Unlike *S. meliloti* Rm1021 it therefore appeared that growth phase was having an effect on the ability of succinoglycan to induce aggregation and subsequent destabilisation of the cultures. Eboigbodin *et al.* (2005) attributed differences in destabilisation by the polymer at the different growth phases to changes in cell surface properties (specifically electrokinetic properties) [117]. However in the case of the *E. coli* strain being utilised in that study (AB1157) differences in destabilisation due to growth phase only altered kinetics of destabilisation and the amount of polymer required for destabilisation [117] . In this case MG1655 at exponential phase does not become destabilised in the presence of succinoglycan and remains relatively turbid (Fig. 5-2). It was therefore possible that additional or alternative characteristics of the cells were inhibiting the ability of succinoglycan to induce aggregation in the exponential phase *E. coli*, which is reduced in stationary phase.

One of the phenotypes associated with the onset of stationary phase is the downregulation of the production of flagella [150]. This has been postulated as a way that the bacteria prepare for a sedentary "lifestyle" that may then facilitate aggregate formation when environmental conditions no longer favour exponential growth [28, 150]. It was therefore hypothesised that the reduction of synthesis of flagella in stationary phase could be leading to the differing phenotypes of the late exponential and stationary phase *E. coli* in the presence of succinoglycan.

To test this hypothesis an *E. coli* MG1655 *fliF* mutant was constructed. Motility of the *fliF* mutant and the parent MG1655 in late exponential phase was compared on 0.3% (w/v) soft LB agar plates (Fig. 5-4). This showed that after 24 hours of incubation at 37°C the parent MG1655 had formed a large diffuse colony that covered most of the plate. The *fliF* mutant however formed only a small colony at the point of inoculation, suggesting that the *fliF* mutant was lacking flagella and was non-motile.

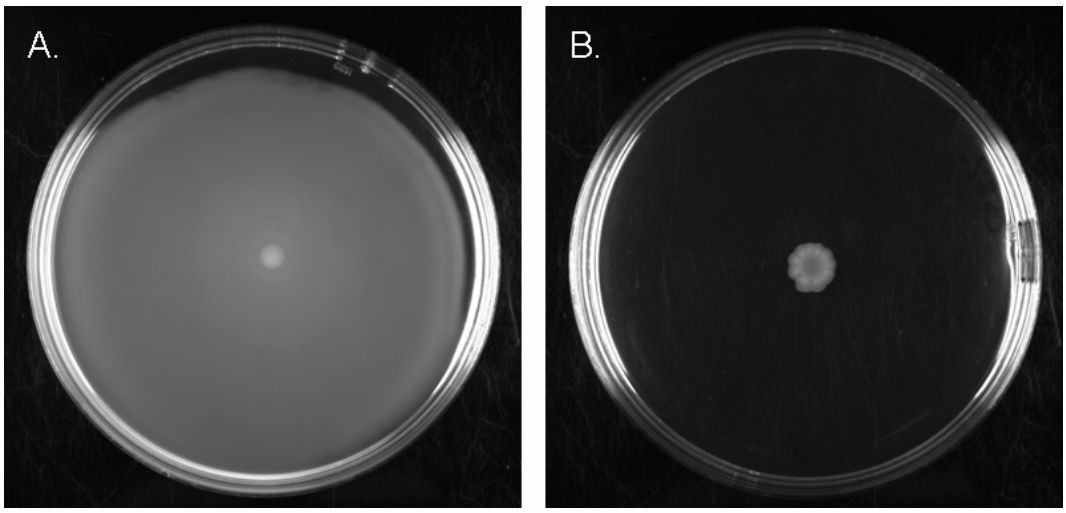


Figure 5-4. Motility of A. *E. coli* MG1655 and B. *E. coli* MG1655 *fliF* mutant. Cultures were grown to exponential phase and 2µl was inoculated into the centre of soft LB agar plates (0.3 % agar w/v). Plates were incubated for 24 hours at 37°C.

To see how the loss of flagella affected the stability of the cultures, the MG1655 *fliF* mutant was grown to late exponential phase and then diluted to various concentrations in the presence of 0.05% (w/v) succinoglycan (Fig. 5-5). Unlike the parent MG1655 in exponential phase the *fliF* mutant destabilised after 24 hours of static incubation in the presence of succinoglycan. The controls that lacked succinoglycan remained as stable, turbid dispersions. Therefore a *fliF* mutant grown to late exponential phase destabilises in the presence of succinoglycan, in contrast to the parent which does not. The cultures were then successively diluted and observed for stability at the different cellular-polymer concentrations. A phase diagram of the diluted cultures showed that the critical polymer concentration decreased with increasing cell concentration, indicative of depletion attraction and as seen for *S. meliloti* and stationary phase *E. coli* MG1655 (Fig.5-6).

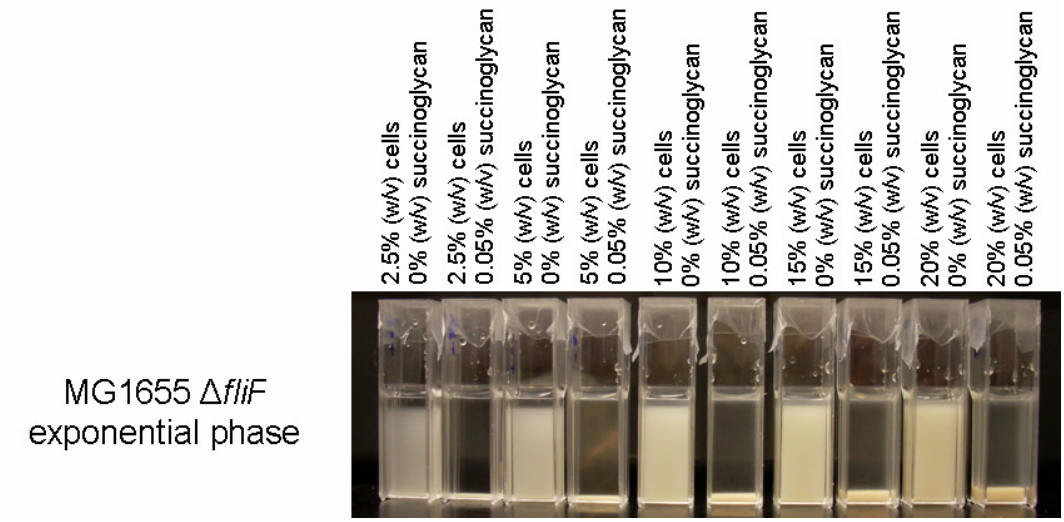


Figure 5-5. Stability of exponential phase MG1655 fliF mutant in the presence of succinoglycan. Cultures were grown to exponential phase in LB, dispersed in PBS at the concentration shown with the concentration of succinoglycan as indicated. Cultures were incubated for 24 hours without shaking before observation of sedimentation.

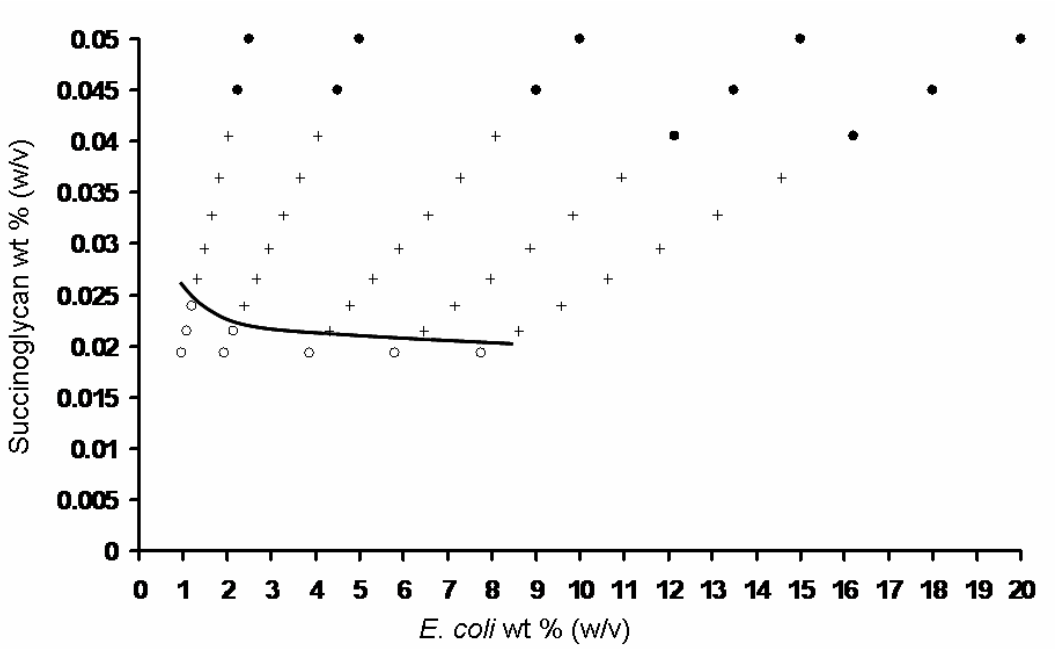


Figure 5-6. Phase diagram of exponential phase *E. coli* MG1655 *fliF* mutant in the presence of succinoglycan. Filled circles are unstable cultures, + are unstable cultures with a turbid upper, open circles represent stable cultures. Line drawn as a guide to the eye showing the transition from unstable to stable cultures.

5. 2 Addition of succinoglycan to polystyrene colloids in water leads to aggregation and destabilisation consistent with depletion attraction

Succinoglycan had been shown to be causing aggregation in two different bacterial systems via depletion attraction. To test if this was also the case in artificial colloids a dispersion of polystyrene colloidal particles was used. In this case the experiments were carried out in deionised water, as in PBS the colloids destabilised without the addition of succinoglycan. This could be a result of screening of the charge on the particles reducing electrostatic repulsion and resulting in loss of stability of the dispersion. To provide a comparison to bacteria, polystyrene particles were chosen that had a similar radius (554nm) to bacteria.

The particles were diluted to various concentrations between 2.5% and 20% (w/v) along with the addition of 0.05% (w/v) succinoglycan. Under these conditions all succinoglycan treated particles had destabilised after 24 hours of static incubation, in contrast to the stable, turbid dispersions with no polysaccharide added (Fig. 5-7). Investigation of the relationship between stability and polysaccharide-particle concentration showed that less polymer was required at higher concentrations of particles for destabilisation to occur, consistent with depletion attraction being the mechanism of aggregation of polystyrene colloids by succinoglycan (Fig. 5-8).

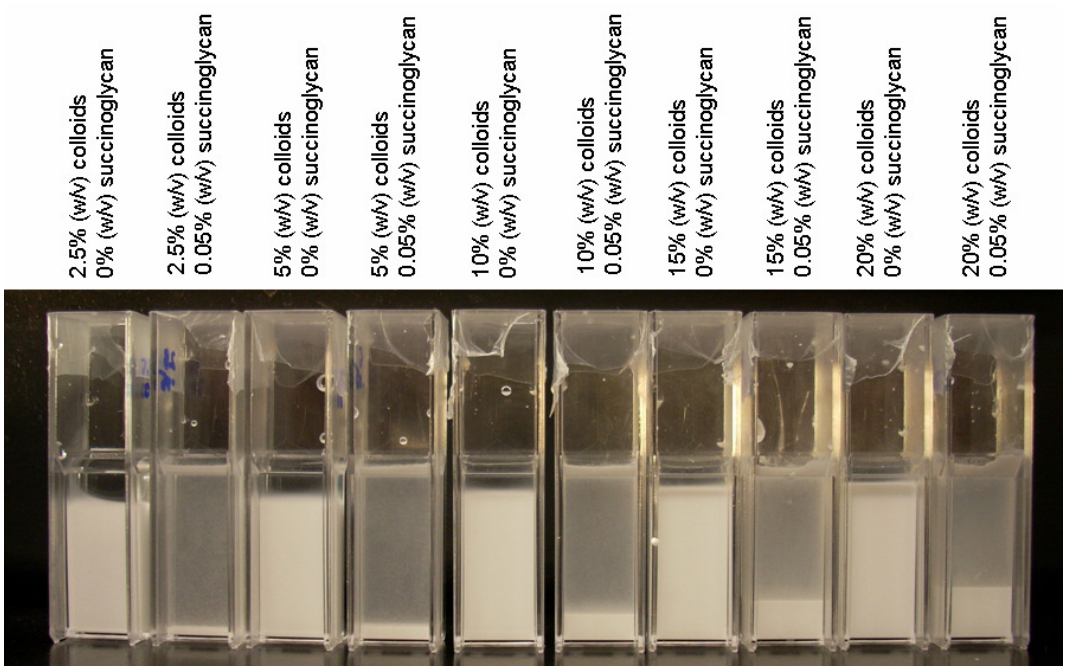


Figure 5-7 Stability of polystyrene colloidal dispersions (r=554nm) in the presence of 0.05% (w/v) succinoglycan. Colloids were dispersed in water at the concentrations given. Dispersions with 0% succinoglycan are controls.

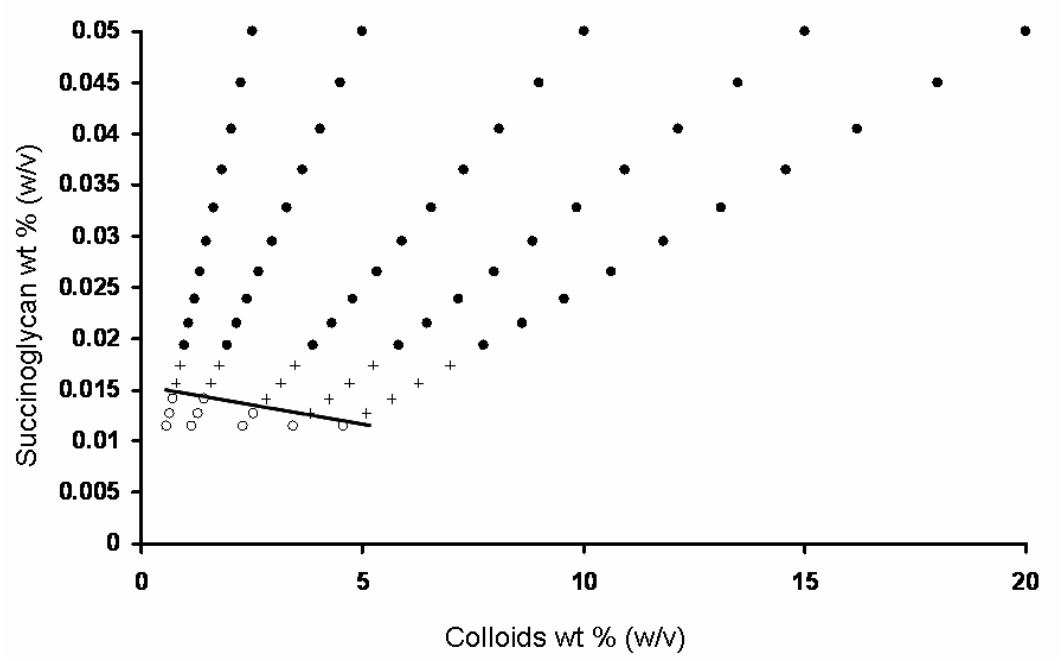


Figure 5-8. Phase diagram of polystyrene colloids in the presence of succinoglycan. Filled circles are unstable dispersion, + are unstable with a turbid upper and open circles represent stable dispersions. Line drawn as a guide to the transition between stable and unstable dispersions.

5.3 Addition of alternative polymers sodium polystyrene sulfonate and xanthan to *S. meliloti* results in destabilisation of the cultures

It therefore appeared that the presence of succinoglycan was able to induce depletion attraction not only in *S. meliloti* cultures but also in other systems such as *E. coli* cultures and polystyrene colloidal dispersions. This raised the possibility that despite the predominance of bridging flocculation as the main hypothesis for the mechanism of bacterial aggregation, depletion attraction in bacterial cultures by polymers could be widespread.

To test this further two alternative polymers were employed to find their effect on cultures of *S. meliloti*. Sodium polystyrene sulfonate (Poly(4-vinylbenzenesulfonate sodium salt) / PSS) has been used as a non-adsorbing polymer in studies of depletion between colloidal particles [151] and between bacterial cells [117]. Also being used in this study is xanthan. Xanthan is composed of pentasaccharide subunits made up of a cellulose backbone (β- 1,4- linked D-glucose residues) with trisaccharide side chains composed of mannose-(β-1,4)-glucuronic acid-(β-1,2)-mannose attached to alternate glucose residues in the backbone by α-1, 3 linkages [56]. The mannose residues are modified to varying degrees by pyruvate and acetyl substituents [56]. Xanthan is biosynthesised by *Xanthomonas campestris*, an organism also associated with plants, although it is a pathogen rather than a symbiont.

5.3.1 Addition of PSS to S. meliloti cultures causes destabilisation of the cultures but not necessarily via depletion attraction

As in the above experiments late exponential phase *S .meliloti* Rm1021 *exoY* cells were diluted to concentrations from 2.5% to 20% (w/v) in water and also PBS in the presence of the polymer, PSS. Compared to the succinoglycan treatments a far greater concentration of PSS was required to induce destabilisation of the cultures in either PBS (Fig. 5-9A) or water (Fig. 5-9B); 0.5% (w/v) of PSS rather than the 0.05% used in the succinoglycan assays. The thickness of the layer formed by the sedimented cells with the addition of PSS was also far larger than the that formed by the sedimented cells in the succinoglycan treatments. As before cultures were observed for stability after 24 hours of static incubation. Successive dilutions allowed the quantification of the critical polymer concentration needed for destabilisation to occur across the range of cell concentrations. Visual observation of the stability of the cultures after dilution was carried out as before. Unlike what had been seen with the addition of succinoglycan, the critical concentration of PSS required to destabilise the cultures and enhance sedimentation did not decrease with increasing cell concentration, in either PBS (Fig. 5-10) or water (Fig. 5-11). The addition of PSS to cells dispersed in PBS did not follow the same pattern as the succinoglycan treatments. The transition line between the stable and unstable states, denoting the critical polymer concentration needed for destabilisation was a level line (Fig. 5-10). This suggests that the polymer concentration needed for destabilisation of the cultures did not decrease with increasing cell concentration. In water it was striking that the opposite is true; the polymer required to destabilise the cultures actually increased with higher concentrations of cells (Fig. 5-11). As the cultures

were diluted the treatments with higher cell concentration stabilised first, in contrast to what had been seen with the succinoglycan treatments.

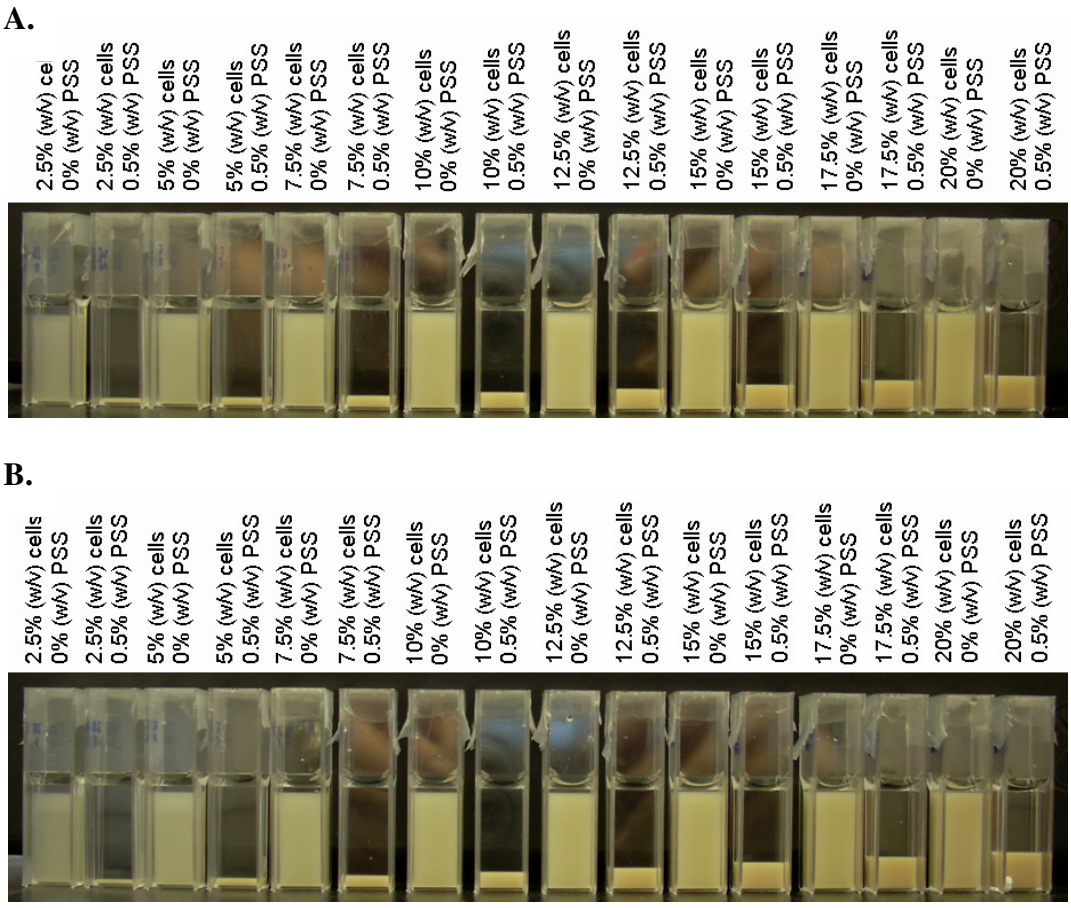


Figure 5-9. Stability of *S. meliloti* in presence of PSS. Cells were diluted to concentrations indicated. PSS was added at a concentration of 0.05% (w/v). Cuvette with 0% PSS were controls. Cultures were incubated for 24 hours without shaking before observing sedimentation. A. PBS treatments. B. Water treatments.

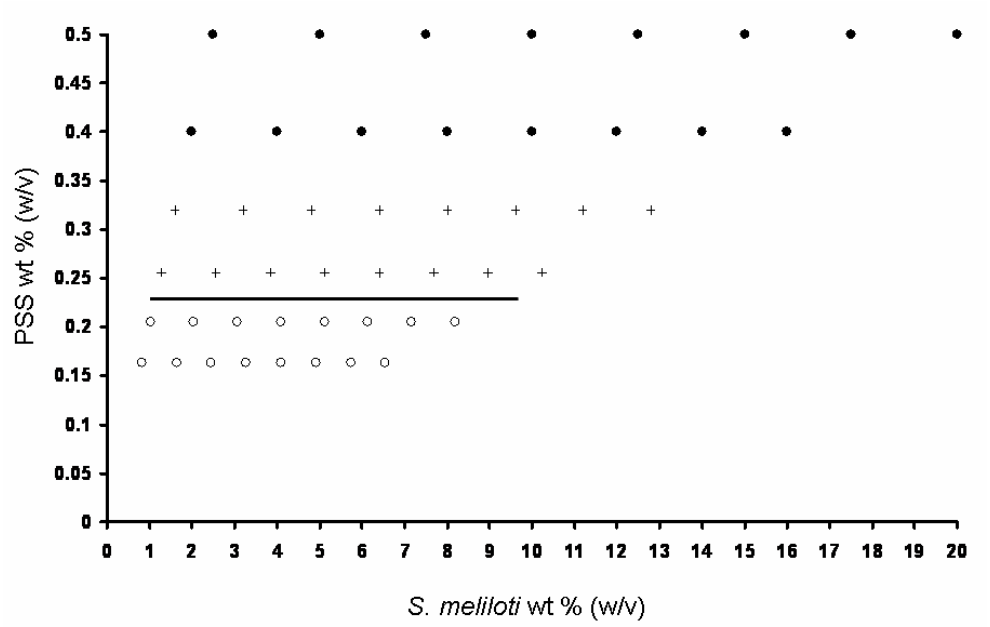


Figure 5-10. Phase diagram of *S. meliloti* in the presence of PSS dispersed in PBS. Filled circles are unstable, + represents unstable but with a turbid upper and open circles are stable cultures. Line drawn as a guide to the eye to illustrate the concentration at which the cultures alter from unstable to stable dispersions.

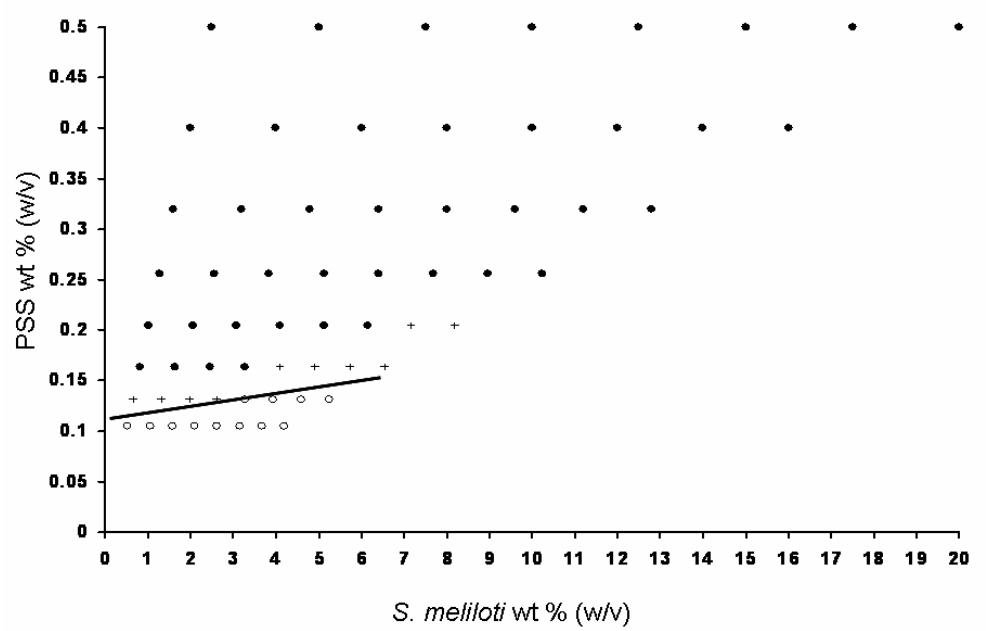


Figure 5-11. Phase diagram of *S. meliloti* in the presence of PSS dispersed in water. Filled circles are unstable, + represents unstable but with a turbid upper and open circles are stable cultures. Line drawn as a guide to the eye to illustrate the concentration at which the cultures alter from unstable to stable dispersions.

5.3.2 Addition of xanthan to S. meliloti cultures causes destabilisation of the cultures by depletion attraction in PBS but not in water

Not only is xanthan also a bacterial polysaccharide but there are similarities between xanthan and succinoglycan in terms of structure, such as both carrying acidic groups and acetyl modifications. It was therefore hypothesised that xanthan would give similar results to succinoglycan.

In PBS at least this hypothesis seems to be confirmed. In contrast to PSS, a lower concentration of xanthan was needed to destabilise *S. meliloti* cultures, similar to succinoglycan (Fig. 5-12). The sediment formed by the cells at the bottom of the cuvette was also compacted to a similar degree as that seen in *S. meliloti* cultures in the presence of succinoglycan, in contrast to the sediment formed in the presence of PSS (compare Fig. 5-12 with Fig 5-9A). Successive dilutions of the cultures determined that the critical polymer concentration for destabilisation decreased with increasing cell concentration (Fig. 5-13). This therefore suggests that much like succinoglycan, xanthan is able to aggregate cells and cause destabilisation of cultures by depletion attraction.

The xanthan treatments in water did not give the same result. The concentration of xanthan required to destabilise the *S. meliloti* cultures was similar to that seen in PBS (Fig. 5-14 and 5-15). However observation of the sedimentation of the cultures after successive dilution determined that the transition line between stability and instability at the critical polymer concentration did not show a dependence on cell concentration (Fig 5-15). However as had been seen with succinoglycan the critical xanthan concentration for instability was lower in water than in PBS.

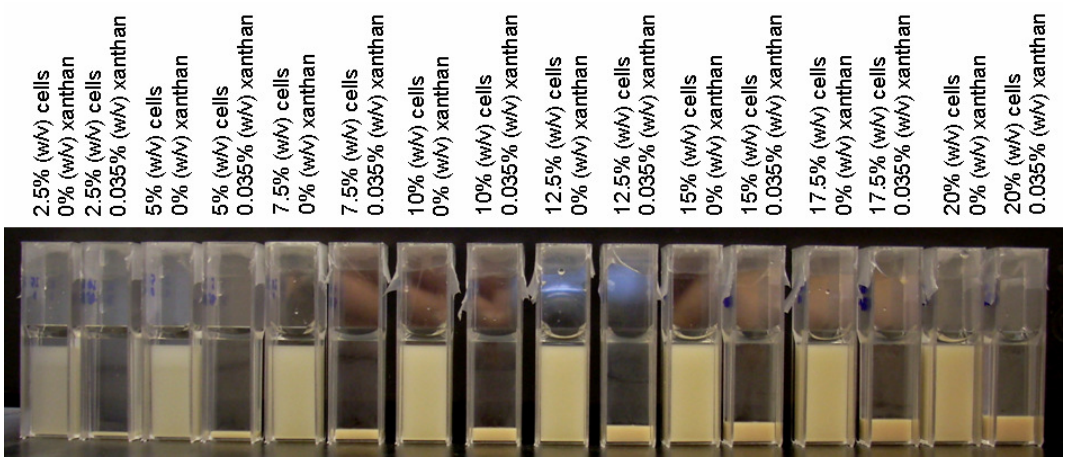


Figure 5-12. Addition of xanthan to *S. meliloti* Rm1021 *exoY* mutant cells dispersed in PBS. Concentrations are as indicated. Cells were incubated for 24 hours without shaking before observation.

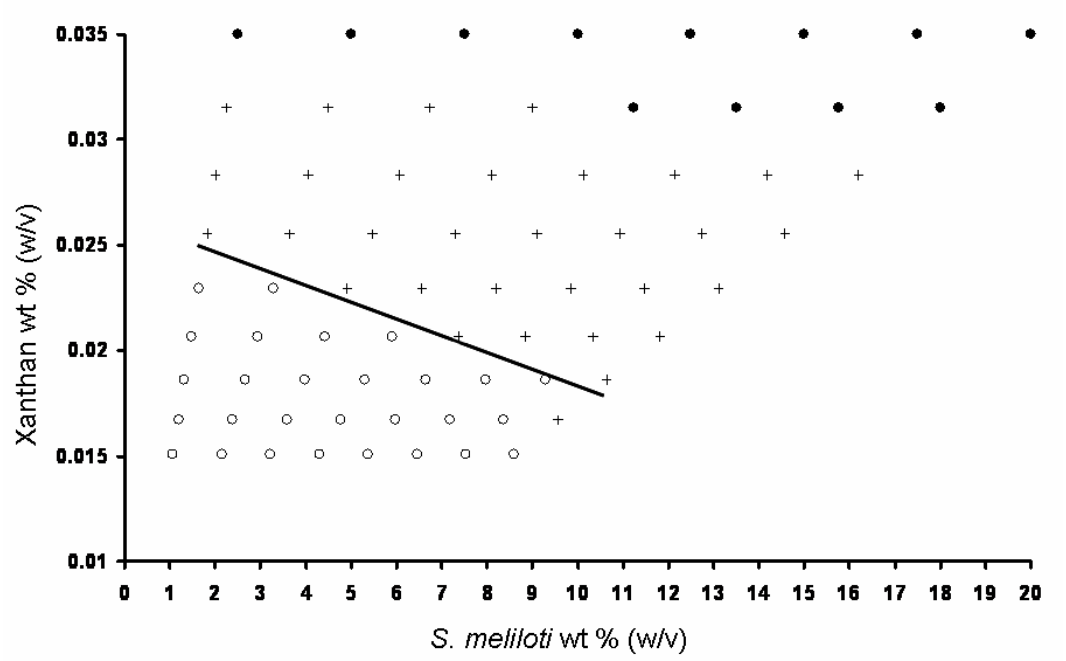


Figure 5-13. Phase diagram of *S. meliloti*- xanthan cultures in PBS. Line represents the critical polymer concentration for the transition between unstable and stable cultures. Filled circles are unstable, + are unstable with a turbid upper, open circles represent stable cultures.

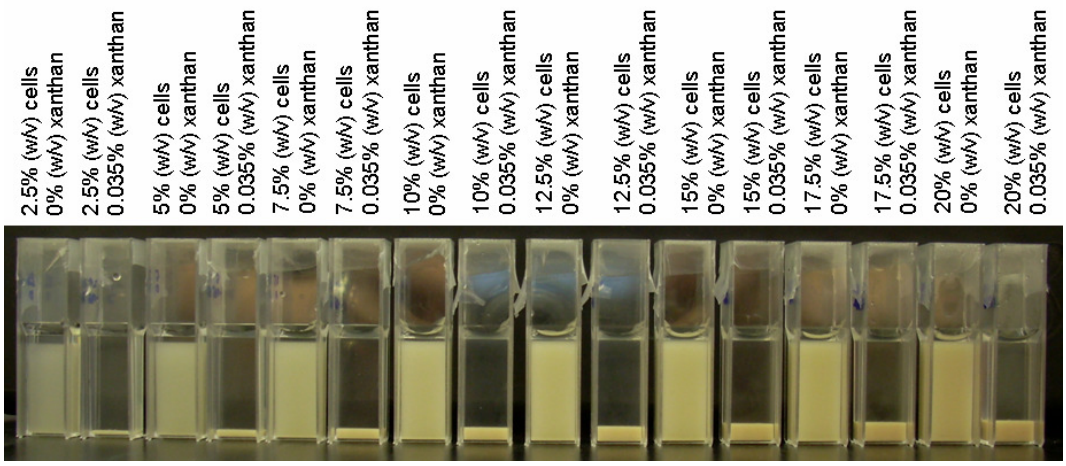


Figure 5-14. Addition of xanthan to *S. meliloti* Rm1021 *exoY* mutant cells dispersed in water. Concentrations are as indicated. Cells were incubated for 24 hours without shaking before observation.

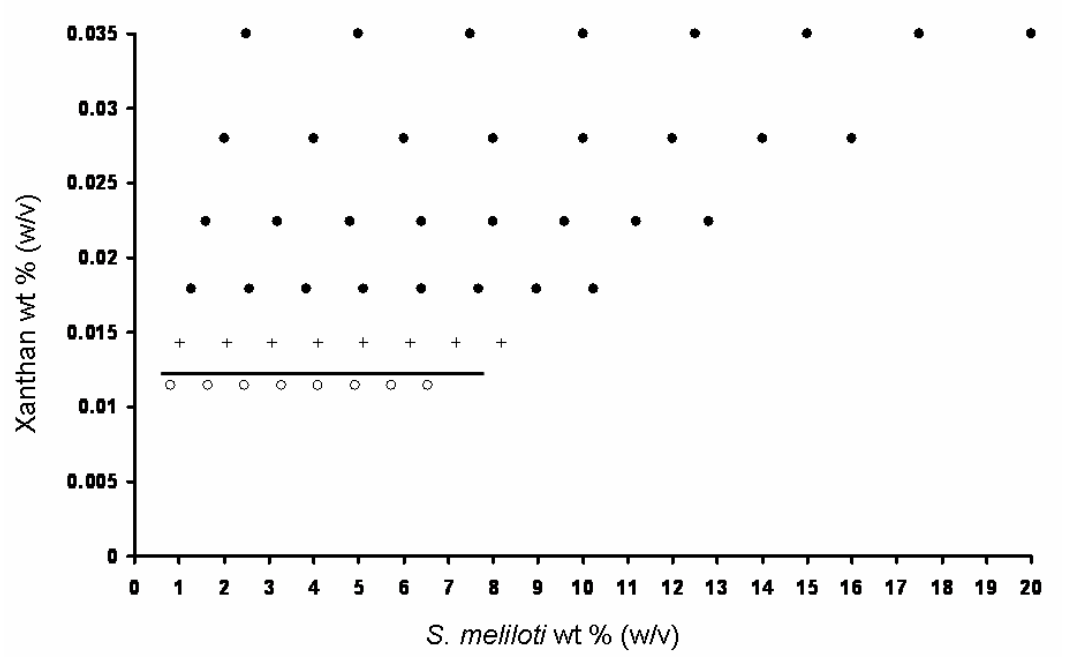


Figure 5-15. Phase diagram of *S. meliloti*- xanthan cultures in PBS. Line represents the critical polymer concentration for the transition between unstable and stable cultures. Filled circles are unstable, + are unstable with a turbid upper, open circles represent stable cultures.

5.4. Discussion

After finding that succinoglycan causes aggregation of bacteria via depletion attraction it was of some interest to find out if this was unique to *S. meliloti* cells and further whether alternative polymers could also result in depletion attraction in *S. meliloti* cultures.

5.4.1 Application of succinoglycan to E. coli *MG1655 and polystyrene colloids*

Succinoglycan was added to cultures of *E. coli* MG1655 and also dispersions of polystyrene colloids. In contrast to *S. meliloti* Rm1021, the effect of succinoglycan on *E. coli* MG1655 was dependent on growth phase. Stationary phase cells of *E. coli* destabilised in the presence of succinoglycan in the same way as had been seen with *S. meliloti*, but late exponential phase cells did not show the same pattern. In late exponential phase it appears that succinoglycan does have some effect, slightly enhancing the rate of sedimentation. However the exponential phase cells had not fully sedimented after 24 hours; it appears that the cells are able to retain stability in the presence of succinoglycan. Many changes occur to the cells on the transition from exponential to stationary phase; one of the most major is the downregulation of flagella synthesis [152]. A non-motile *E. coli* MG1655 *fliF* mutant, which was then harvested in late exponential phase and incubated with succinoglycan, did destabilise. This suggests that in *E. coli* MG1655 the presence of flagella can inhibit succinoglycan-driven depletion attraction. It is interesting that this was not seen in *S. meliloti* Rm1021 late exponential phase cells. This could be due to differences in motility and production of flagella between the two species. As stated in the previous chapter microscopic observation of *S. meliloti* Rm1021 cells in late exponential

phase showed few of the cells are motile. A lack of flagella was also seen in TEM preparations of the parent strain *S. meliloti* Rm1021. This suggests that compared to *E. coli* MG1655 *S. meliloti* Rm1021 is relatively lacking in cells expressing flagella and is less motile. It is difficult from these experiments to tell if the presence of flagella or actually motility is required for the cells to overcome aggregation by depletion attraction. In the presence of supernatant from the *exoS* mutant it was observed that a very low number of motile cells were able to swim freely between aggregates suggesting motility can be used to overcome depletion attraction and aggregation. However in the case of exponential *E. coli* MG1655 cells in the presence of succinoglycan no motility was observed despite the lack of aggregation and destabilisation. This suggests that perhaps the presence of flagella could provide a steric hindrance to depletion attraction. Future experiments could utilise *E. coli* mutants that are flagellated but non-motile to see if they also retain stability in the presence of succinoglycan.

Investigation of the polymer concentration needed for destabilisation of the stationary phase *E. coli* and late exponential phase *E. coli fliF* mutant, showed that the critical polymer concentration decreased with increasing cell concentration consistent with depletion attraction. This therefore suggests that in the absence of flagella, *E. coli* MG1655 also aggregates in the presence of succinoglycan the same as *S. meliloti* Rm1021. The same result was achieved with polystyrene colloids. Comparison with the results obtained for *S. meliloti* showed that similar concentrations of succinoglycan were required to induce depletion attraction in both *E. coli* MG1655 and the polystyrene colloids. This suggests that the depletion attraction driven by succinoglycan is not unique to *S. meliloti* or even bacteria and

can drive aggregation in a number of colloidal systems. This raises interesting questions as to the functional role that succinoglycan may play in the rhizosphere of the plants that *S. meliloti* occupies in its natural environment. The rhizosphere is a complex and also potentially crowded environment, with many microniches composed of multiple species of bacteria, plant and organic matter and colloidal material from the soil. The production of succinoglycan secreted into the environment by *S. meliloti* may therefore have an impact on the aggregation and organisation of this complex environment.

5.4.2 Addition of alternative polymers to S. meliloti cultures

Whilst the role of succinoglycan as a depletion agent seemed clear it was also of some interest to find out whether other polymers could also cause aggregation of bacterial cultures via depletion attraction. Two polymers were tested on *S. meliloti* cultures; PSS a polymer used in depletion experiments on colloids and on *E. coli* AB1157 [117] and xanthan. Xanthan was chosen as it is also a microbial exopolysaccharide (produced by *X. campestris*), it is commercially available and as mentioned above, shares properties with succinoglycan [42].

The addition of PSS did result in destabilisation of *S. meliloti* cultures. However it was unclear from the experiments looking at destabilisation of cultures with different concentrations of polymer and bacteria whether this was due to depletion attraction, in either PBS or water. In water particularly the critical concentration of polymer needed for destabilisation actually increased with cell concentration, the opposite of what would be expected with depletion attraction. PSS had previously been reported to cause destabilisation of *E. coli* AB1157 cultures by depletion attraction [117, 148]. In the case of Eboigbodin *et al.* all of those experiments

were carried out in water. The phase diagrams in the case of *E. coli* AB1157 were the same as found in this study for *S. meliloti*-PSS in water, with the critical polymer concentration increasing with cell concentration [117]. However despite the upward trend for the critical polymer concentration in relation to cell concentration the authors still suggested that depletion attraction was the force behind destabilisation of *E. coli*- PSS cultures [117]. Given that depletion attraction is a crowding mechanism it seem unlikely that this is the mechanism behind the destabilisation. Schwarz-Linek et al. found that *E. coli* AB1157 in buffered solutions gave similar results as those seen in this study with *S. meliloti* in PBS, which was attributed to depletion attraction [148]. As with succinoglycan, the differing results may be explained by conformation of the polymer in water and buffered solutions. It seems probable that in water PSS is causing destabilisation by bridging flocculation. In water there will be no screening of the charges carried on the polymer or the cell surface. While the cell surface has an overall negative charge it is possible that there may be pockets of positive charge that the coiled polymer, by taking on a 'loopy' configuration may be able to interact with, resulting in absorption [148]. This would only occur in water due to the lack of screening of the charges and due to the coiled conformation of the polymer. In PBS the screening of the charges would mean such interactions were less likely to occur and the polymer would be non-adsorbing due to the decrease in entropy of the polymer. As mentioned above Schwarz-Linek et al. found a similar pattern of results in buffered solution with *E. coli* AB1157 which was attributed to depletion attraction [148].

Results for addition of PSS with *S. meliloti* may provide further evidence in support of succinoglycan driving aggregation of *S. meliloti* by depletion attraction

studied in the previous chapter. Comparing results from both Eboigbodin et al, Schwarz-Linek et al. and *S. meliloti*-PSS mixtures from this study to those found for *S. meliloti*-succinoglycan mixtures in the previous chapter shows that less succinoglycan is required for destabilisation compared to PSS (~0.01 wt % (w/v) succinoglycan compared to ~0.1% wt % (w/v) PSS in deionised water or PBS) [117, 148]. This again is consistent with the relative depleting ability of succinoglycan rods compared to coil polymers of PSS. Due to the greater effect of rods less succinoglycan is required to induce depletion attraction than PSS. This therefore fits with the model of depletion attraction driving aggregation.

Xanthan also resulted in destabilisation of the cultures in both water and PBS. In water the critical polymer concentration for transition from stability to instability was constant across cell concentration. In PBS however similar results were seen as for succinoglycan; the critical polymer concentration for the transition between stability and instability decreased with increased cell concentration. Xanthan can therefore also cause aggregation of bacteria via depletion attraction. This shows that another microbial extracellular polysaccharide is capable of inducing bacterial aggregation via depletion attraction. Xanthan has been shown to be essential to the formation of aggregates in *X. campestris* [21]. However the aggregates were shown to be encased in an extracellular matrix by SEM and the cells within the aggregates were resistant to dispersal by vortexing [21]. This is indicative of bridging rather than depletion attraction. Intriguingly however the authors also found that dispersal of the aggregates was possible by production of an extracellular enzyme identified as endo-β1,4-mannanase [21]. This enzyme had no activity against xanthan and raised the possibility that while xanthan was essential for the formation of aggregates, another

unidentified polysaccharide was also needed [21]. It could be possible therefore that xanthan may drive aggregation of the cells by depletion attraction but the aggregates are then stabilised by production of a second adsorbing polysaccharide.

In conclusion it has been shown that the addition of succinoglycan can result in depletion attraction in other systems to *S. meliloti*, such as *E. coli* cultures and polystyrene colloidal dispersions. Depletion attraction may be a more widespread phenomenon than currently realised with other bacterial polysaccharides such as xanthan and manmade polymers such as PSS also able to result in depletion attraction of bacterial cultures.

Chapter 6

Surface attachment in *S. meliloti* Rm1021

In the previous chapters it has been shown that succinoglycan, when present in high enough amounts, causes *S. meliloti* Rm1021 cells to aggregate by a mechanism of depletion attraction. This chapter now focuses on surface attachment of *S. meliloti* Rm1021 and the role of succinoglycan in this process. In studies of bacterial aggregation the emphasis is often placed not on just cell-cell aggregation but also the ability of the cells to adhere to a surface. While the definition of biofilm has now been extended to include cell-cell aggregates, the original definition was that biofilms were accumulations of bacteria attached to a surface [27]. Surface attachment may be an important component of the life history of many bacteria, allowing them to colonise and persist in a favourable niche. Surface-associated biofilms became a focus of study due to the role of these structures in the persistence of bacteria in certain types of disease [28, 30, 31, 37, 40, 153-155]. The increased study of surface-associated biofilms led to the realisation that they are ubiquitous and could represent the predominant way that bacteria live in their natural environment [28, 155].

The ubiquity of surface-associated bacterial biofilms in the environment has led to the study of surface attachment of not only pathogenic bacteria that cause disease in humans but a range of species, including those that interact with plants. This includes plant pathogens such as *A. tumefaciens* and *X. campestris* or symbionts such as *R. leguminosarum*, all of which can affect various crop yields. *S. meliloti* was unstudied before this investigation, but since then work has also been published concerning *S. meliloti* surface-associated biofilms [95, 99, 132]. Exopolysaccharide

production by these species has been found to play a critical role in surface-associated biofilm formation [21, 98, 156]. In *R. leguminosarum*, which forms a symbiotic association fixing nitrogen for pea plants, mutations that abolish exopolysaccharide production also led to the cells being unable to form surface-associated biofilms [98]. Similarly *A. tumefaciens* mutants that have decreased cellulose production have reduced surface-associated biofilm formation; mutants that overproduce cellulose have increased biofilm formation compared to wild type [18]. Aggregation and surface attachment of biofilms in *X. campestris* appears somewhat more complicated as discussed in the previous chapter. However it has been found that in minimal media the upregulation of xanthan biosynthesis leads to increased surface-attached biofilm formation in the wild type strain; further *gumB* mutants that abolish xanthan production also have disrupted surface-attached biofilm formation [22]. Due to the role of succinoglycan in aggregation of cells, the aim of this chapter is to therefore investigate the role of succinoglycan in the formation of surface-attached biofilms in *S. meliloti* Rm1021 in the context of depletion attraction. Many surface attachment studies also focus on the role of surface-associated biofilms in the persistence of the bacteria in their free-living form and how the bacteria regulate surface-attached biofilm formation in relation to virulence [21, 98, 156]. It is hypothesised that biofilm formation provides bacteria with protection from environmental stresses but means a reduced capacity for growth and reproduction. This trade-off must therefore be carefully controlled by the bacterium to ensure evolutionary success. This will also be discussed in *S. meliloti* and how ExoS may have a role in regulating this trade-off. During the course of this study various other investigators have also studied biofilms in *S. meliloti* and found similar results [95, 132].

6.1. The *exoS* mutant forms ordered biofilm-like structures in LB_{MC} similar to those formed by other species

The upregulation of succinoglycan biosynthesis in *S. meliloti* Rm1021, either by introduction of the *exoS* mutation or by growth in minimal media, leads to an increase in aggregation of this strain. It therefore seemed likely that these conditions would also increase the attachment of *S. meliloti* Rm1021 cells to a surface. In previous studies on *R. leguminosarum* and *X. campestris*, chambered coverslides in conjunction with green fluorescent protein (GFP) labelled cells were used in confocal microscopy to investigate the structure of the surface-associated structures formed by these species [22, 98]. This was an ideal set-up with which to study the *S. meliloti* system. The chambered coverslides consist of chambers mounted on a coverslip which forms the base of the chamber (Fig. 5-1A and 5-1B). By using an inverted microscope the coverslip and the bacteria that are on that coverslip can then be imaged (Fig 5-1A and 5-1B). In *R. leguminosarum* and *X. campestris* this allowed imaging of the GFP labelled cells which formed surface-associated biofilm structures on the coverslip at the base of the chamber [22, 98]. In the case of *S. meliloti* this system allowed visualisation of the sediment that is formed by the bacteria during static incubation, which as shown previously is enhanced in the *exoS* mutant due to formation of aggregates by depletion attraction.

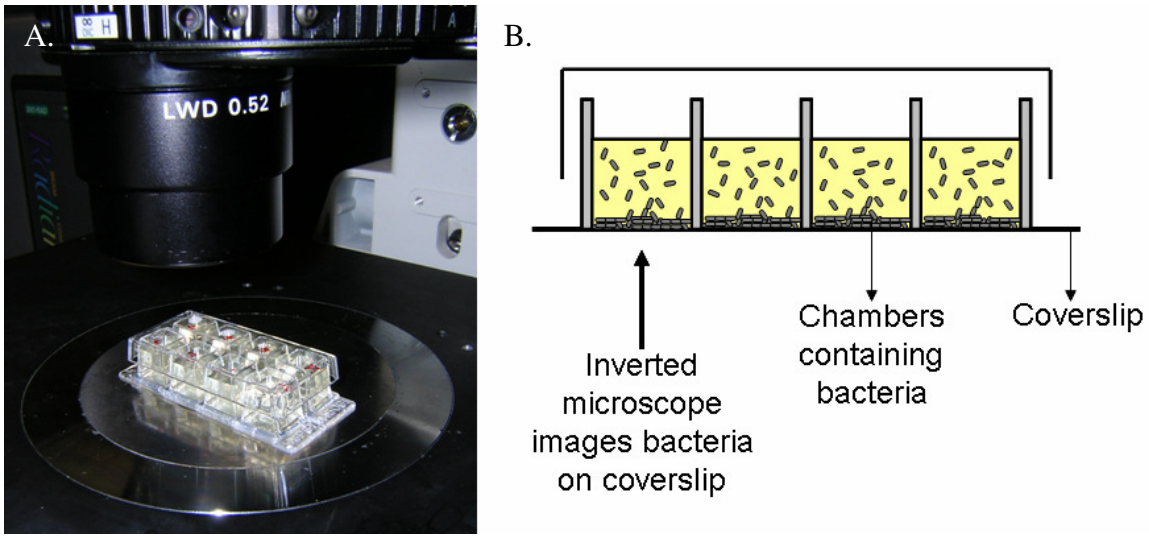


Figure 6-1 Chambered coverslides used for imaging of the sediment formed by *S. meliloti*. A. Photograph of the chambered coverslide with samples mounted on confocal microscope stage. B. Diagram of the chambered coverslides showing the important features.

The previous chapters have shown that in LB_{MC} the *exoS* mutant has an enhanced rate of sedimentation as a result of the formation of aggregates by depletion attraction. In order to image the *exoS* mutant cells in the sediment using confocal microscopy as outlined above, the pHC60 plasmid, which results in constitutive production of GFP in *S. meliloti* was mobilised into the *exoS* mutant by triparental mating. In order to compare the effects of succinoglycan production and loss of flagella on the structure of the sediment formed by the bacteria, the plasmid was also mobilised into the parent strain Rm1021 and the *exoY*, *exoSexoY* and Δ*fliF* mutants. The pHC60 plasmid has been used to study bacteria *in planta*, and has been engineered so that it is stably maintained in the absence of antibiotic selection [123]. This was advantageous as antibiotics were not being used in these experiments due to their possible effect on biofilm structure (Downie, J. A; personal communication). The growth of the strains carrying the pHC60 plasmid was similar to the growth rate

of the strains without the plasmid (Fig. 6-2); the sedimentation of the strains was the same as seen in the strains not carrying the plasmid.

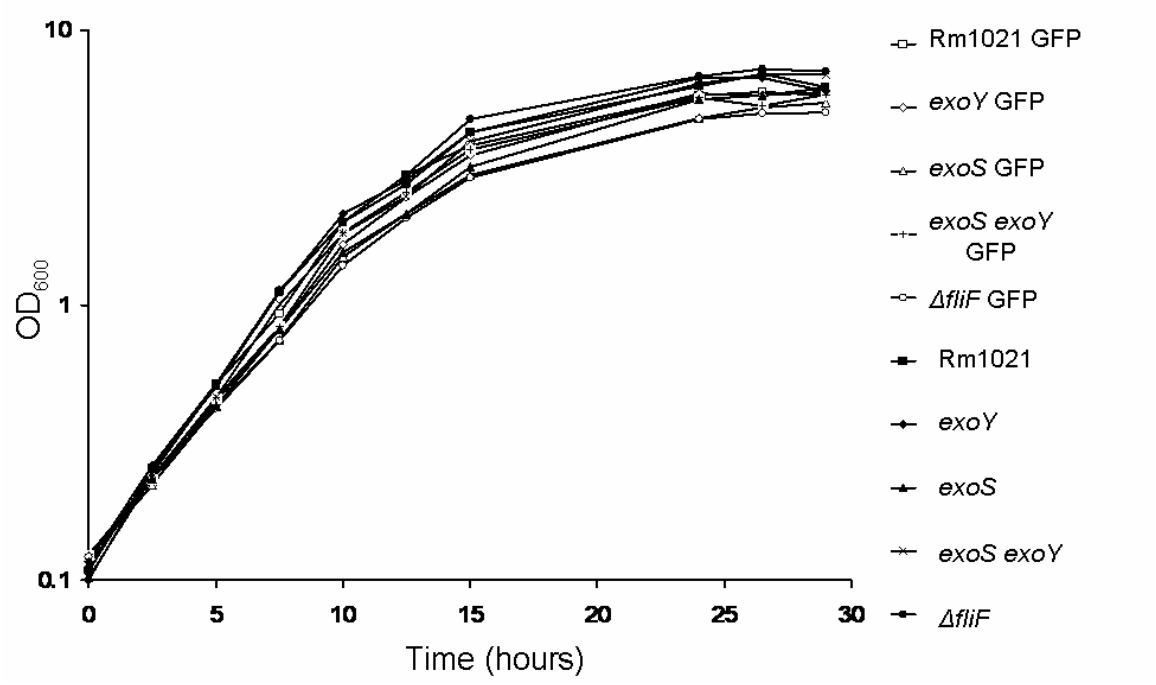


Figure 6-2. Comparison of the growth curves of the *S. meliloti* strains and the strains when carrying the GFP expressing plasmid pHC60 which results in constitutive production of GFP.

To visualise the structure of the sediment formed by the strains, the GFP-expressing bacteria were grown to late exponential phase in LB_{MC} and allowed to sediment over 24 hours in the chambered coverslides by static incubation, as had been done in the cuvettes in previous experiments. The structures that had formed on the coverslip of the slides were then imaged by an inverted confocal microscope.

The confocal microscopy showed that the *exoS* mutant forms a completely different structure compared to the other strains (Fig 6-3). The sediment, rather than forming a compacted mat of cells, appears to have a more complex structure. This developed structure is similar to biofilms formed by other species (for example see

[22]). The cells are highly compacted. An xz profile of the structure shows the formation of mushroom-shaped structures. As with the microscopy of the aggregates formed in the bulk culture by the *exoS* mutant, the cells also appear to be largely interacting with each other laterally. Interestingly the cells do not completely cover the coverslip surface, instead forming interlinked aggregates with gaps in between them. Such gaps have in other studies of biofilm formation been construed as 'water channels', possibly allowing exchange of nutrients between the biofilm and the bulk fluid [28, 155].

In contrast to the *exoS* mutant, the other strains of *S. meliloti* Rm1021 appear to have much less obvious structure, with the cells forming a diffuse mat over the entire coverslip (Fig. 6-3). The xz profiles of the sediment formed by these strains also illustrates this diffuse mat-like appearance. When viewed under the microscope, the cells were visibly subject to Brownian motion, with all of the cells randomly fluctuating in position in the medium. This is different from the *exoS* mutant which showed little movement as the cells are more tightly compacted and aggregated in the *exoS* structure. The *exoY* mutation abolishes production of succinoglycan; the fact that the *exoSexoY* mutant does not have the same structure as the *exoS* mutant suggests that as detailed in the previous chapters the formation of this enhanced, complex structure is therefore related to the overproduction of succinoglycan. This is supported by the lack of structure seen in the parent Rm1021 and the Δ*fliF* mutant as in the previous chapters it has been shown that in LB_{MC} Rm1021 is producing little or no succinoglycan (Table 3-2).

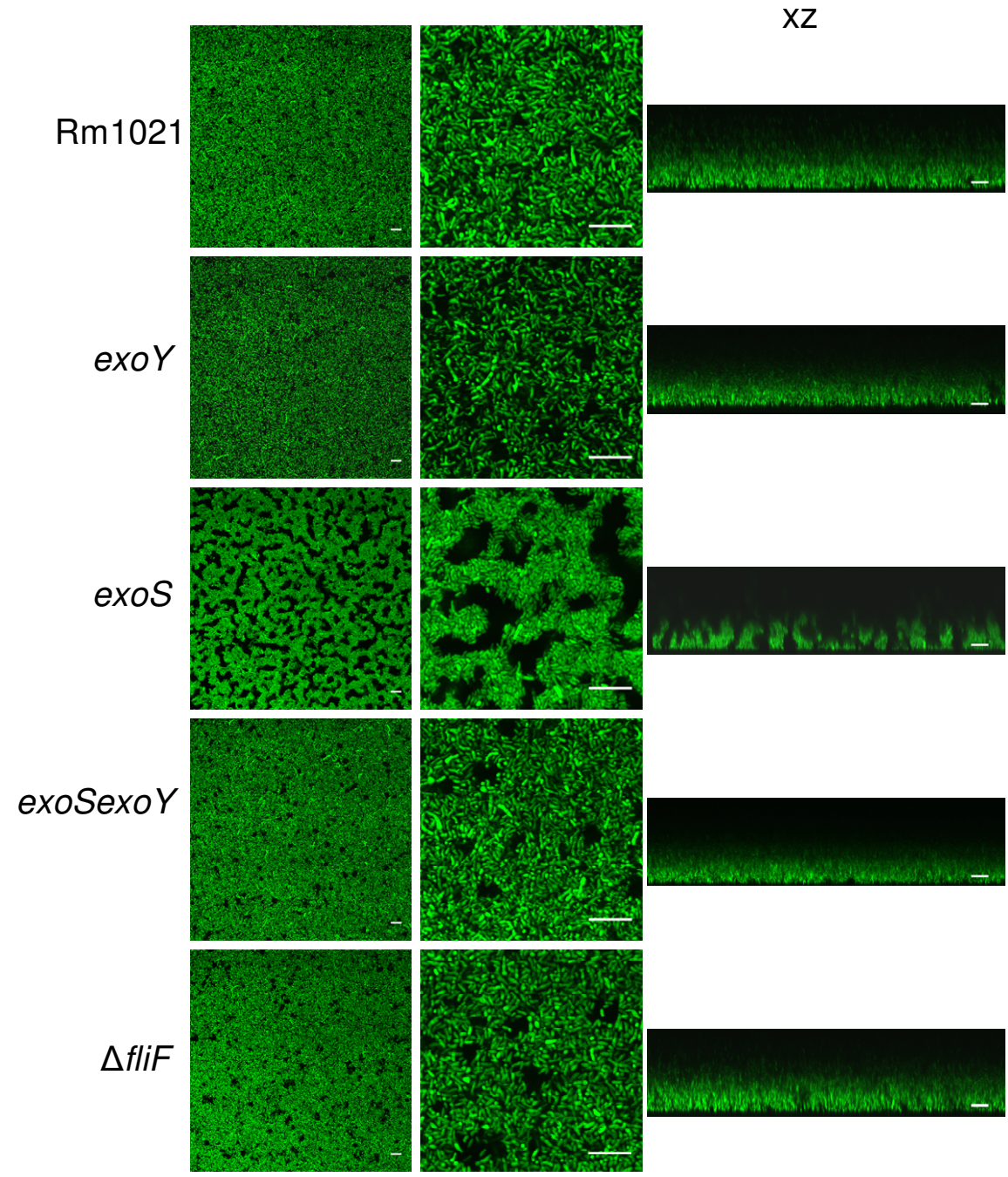


Figure 6-3. Confocal microscopy of the sediment formed by *S. meliloti* strains expressing GFP from the plasmid pHC60. Identity of the strains is shown on the left. xz profiles are side view images of the structures formed. Strains grown to late exponential phase in LB_{MC} and then incubated in chambered coverslides without shaking for 24 hours. Bars= 10μm. Observations repeated in successive experiments.

The complexity of the *exoS* structure was surprising; it could have perhaps been expected that the sedimenting aggregates would have simply formed a mat of cells on the surface, albeit more compacted than the other strains. To confirm that the formation of the surface-associated *exoS* mutant structure was due to the accumulation of sedimenting aggregates the developing structure was monitored over time by confocal microscopy (Fig. 6-4). A layer of aggregates formed in less than four hours. It therefore appeared that the enhanced sedimentation of the *exoS* mutant depletion-induced aggregates was leading to the formation of a surface-associated biofilm-like structure comparable to the biofilms formed by other species. This was further confirmed by dispersing the GFP-expressing strains in filtered *exoS* mutant supernatant (Fig. 6-5). In previous experiments this led to the formation of aggregates and enhanced sedimentation in the parent strain Rm1021 and in the *exoY* and *exoSexoY* mutants as well as the Δ*fliF* mutant (Fig. 3-7 and Fig. 3-8). Imaging of the sediment of these strains showed that the cells were forming a highly compacted, developed biofilm-like structure similar to the *exoS* mutant (Fig. 6-5).

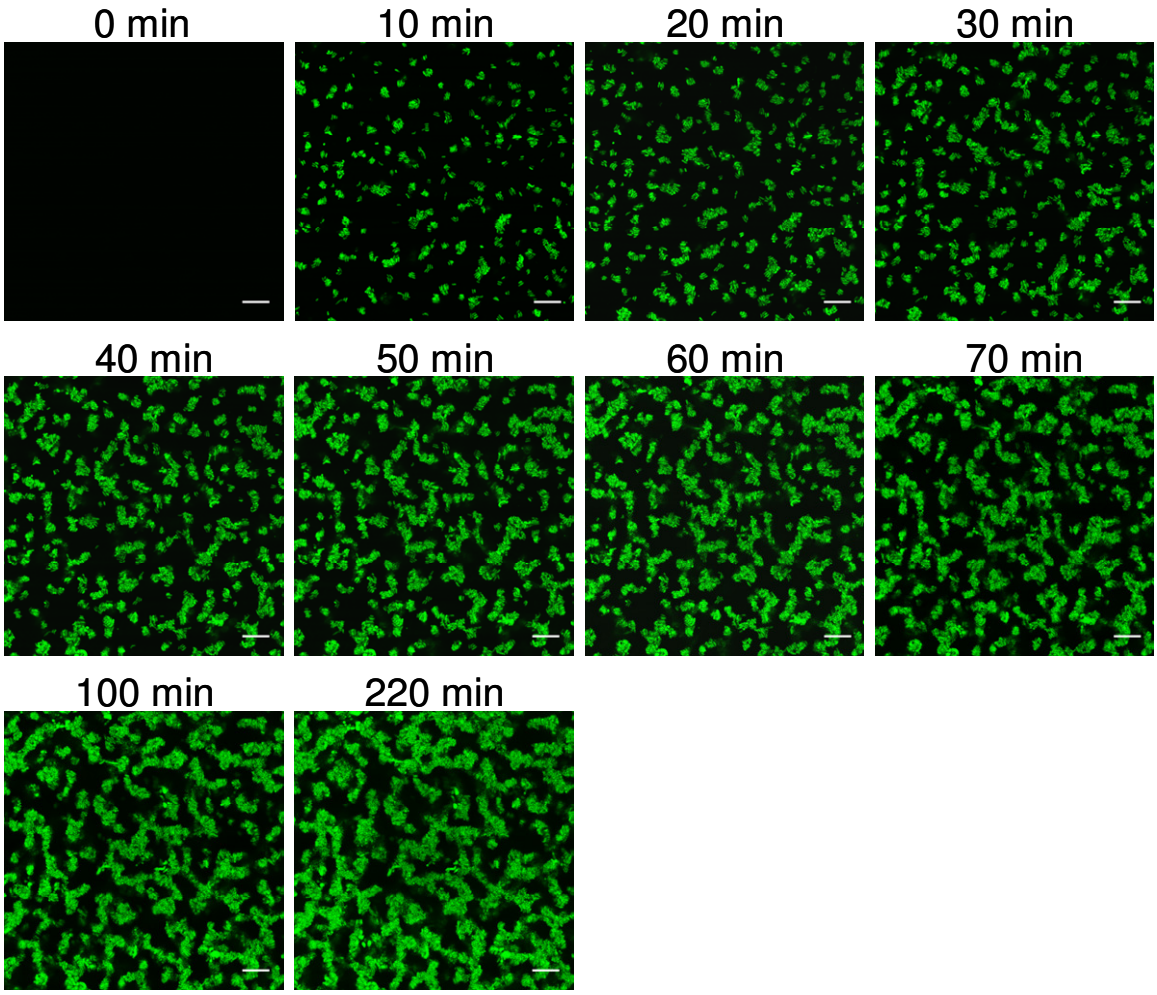


Figure 6-4. Formation of the sediment of the *exoS* mutant, which has a biofilm-like structure similar to that seen in other species, is due to the sedimentation of aggregates of cells. The *exoS* mutant was grown to late exponential phase in LB_{MC} and then incubated in a chambered coverslide for 24 hours without shaking. Above each panel indicates the time at which the image was taken after inoculation of the chambers with the *exoS* mutant expressing GFP. Bars=10μm.

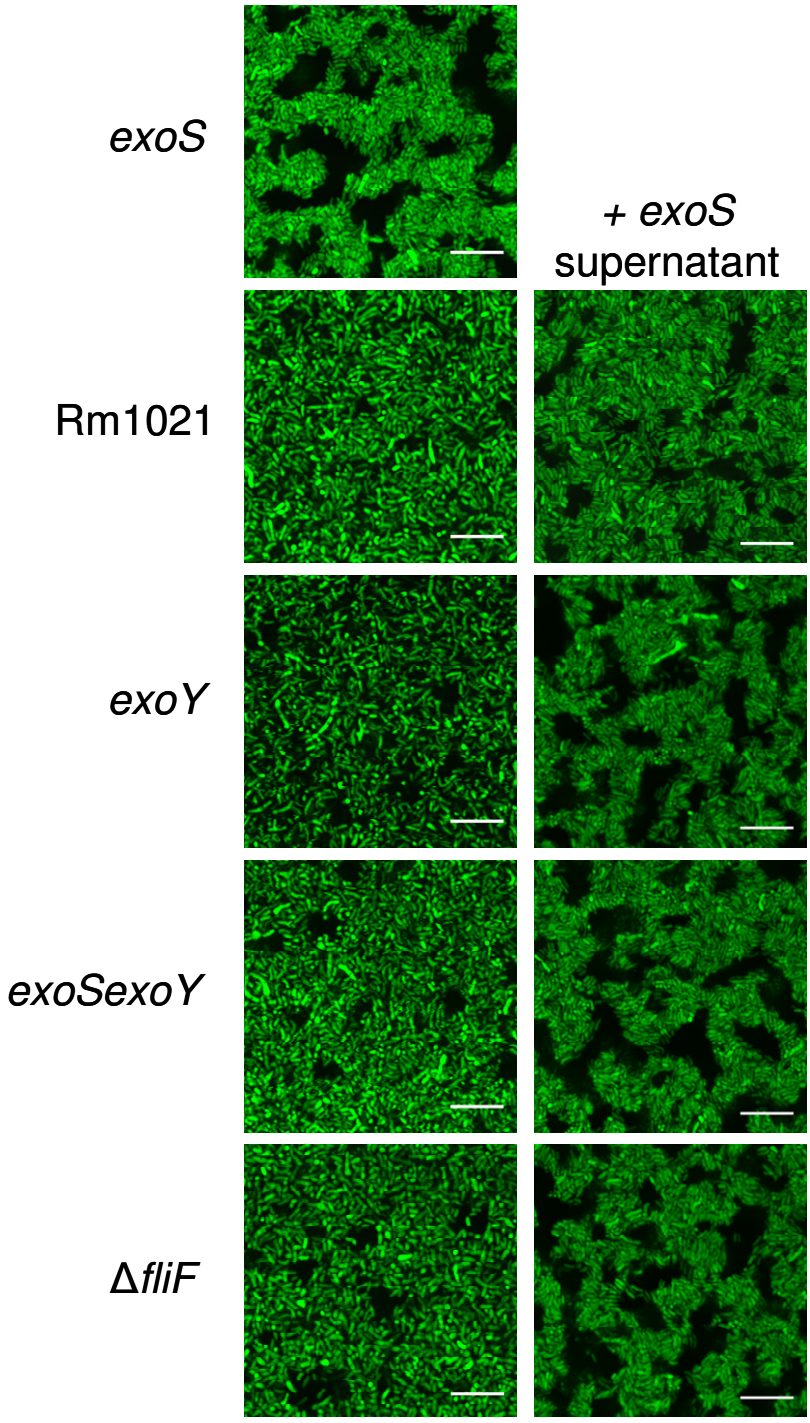


Figure 6-5. Addition of *exoS* supernatant to the other strains of *S. meliloti* results in a sediment that resembles the biofilm-like structure of the *exoS* mutant. Strains grown to late exponential phase in LB_{MC} and then incubated for 24 hours without shaking in chambered coverslides. Bars=10µm.

In other studies of surface-associated biofilm formation the strains are often diluted prior to incubation [30]. To see if this had an effect on biofilm formation the strains were diluted to an OD_{600} of 0.1 before incubating without shaking for 24 hours. This does alter the formation of a surface-associated structure at the base of the chamber on the coverslip, most noticeably in the *exoS* mutant (Fig. 6-6). All the strains, including the *exoS* mutant now almost completely cover the coverslip. However as had been seen in the previous images, the *exoS* mutant still had a more advanced structure. The cells form a compact layer and are not moving by Brownian motion. The structure is also interspersed with small holes or pores. In contrast the parent Rm1021, the *exoY*, *exoSexoY* and Δ*fliF* mutant all form a diffuse mat-like structure on the base of the chamber (Fig. 6-6).

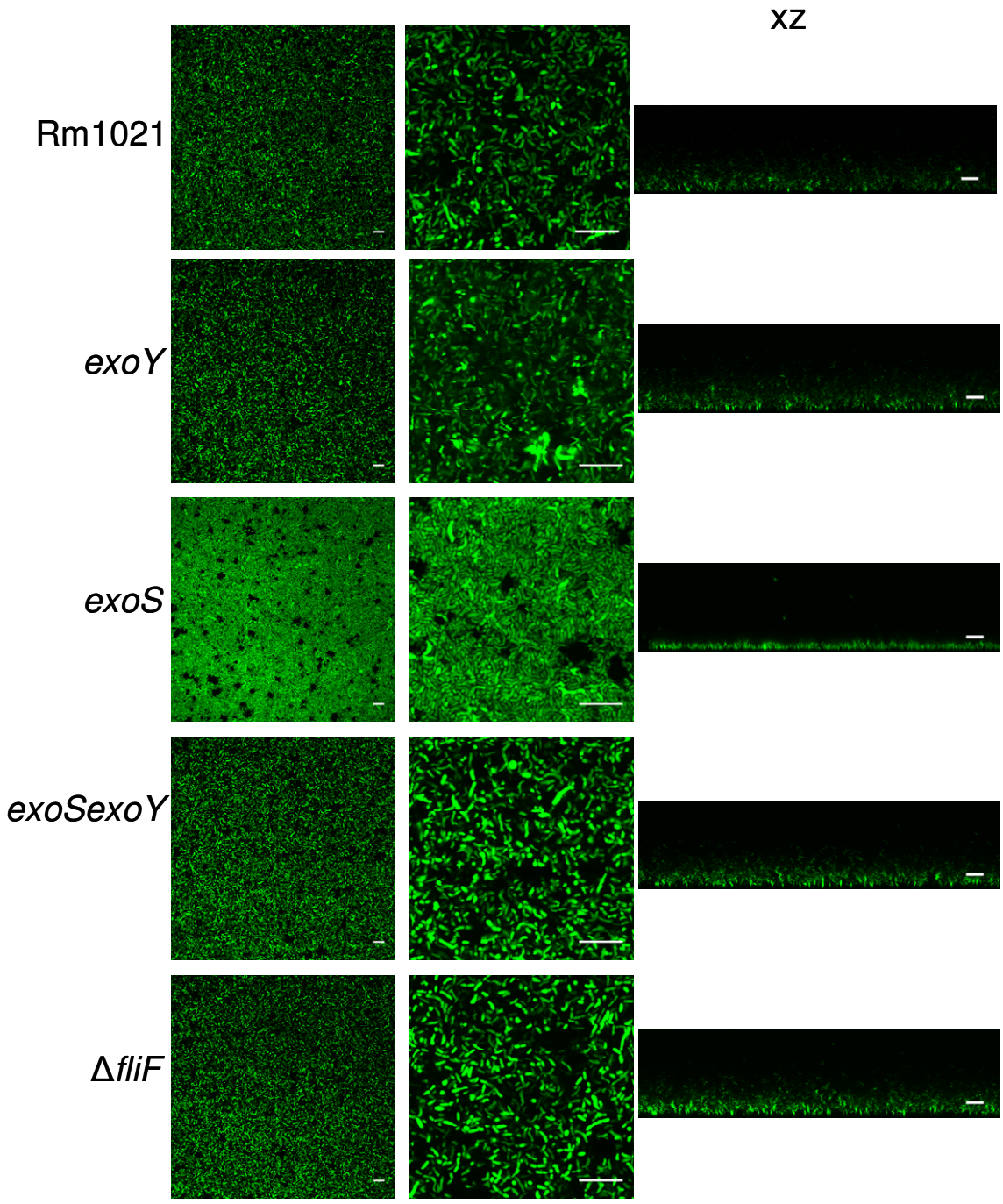


Figure 6-6. Structure of the sediment formed by the *S. meliloti* strains when the strains have been diluted to an OD_{600} 0.1 before being put into the chambered coverslides. Incubated for 24 hours without shaking before imaging. Bars=10μm.

6.2. The *exoS* mutant forms biofilms that are more strongly attached to the surface than other Rm1021 strains

The sedimented *exoS* mutant cells therefore form a developed structure that shares many structural features to those published for surface-associated biofilms formed by other species. However as described in section 4.1.2, one of the features of the depletion induced aggregation and destabilisation of the *exoS* mutant culture is the reversibility of the sedimentation. As such the *exoS* mutant cells are able to be easily redispersed with mixing. The surface-associated biofilm-like structure formed by the sedimentation of the *exoS* mutant is therefore only transiently attached to the surface. In contrast surface-associated biofilms formed by some species have been shown to be attached strongly to the surface and are resistant to removal by shear forces.

6.2.1 Crystal violet assays show the exoS mutant has greater surface attachment than other S. meliloti Rm1021 strains

Therefore to investigate if *S. meliloti* Rm1021 does attach to the surface more strongly, and the effect of succinoglycan production on this process, a crystal violet assay was used. This assay involves incubating the strains in microtitre plates [30]. Bacteria not attached to the surface are then removed from the wells by washing, and the bacteria that remain are stained with crystal violet. The crystal violet can then be solubilised by addition of acetic acid and the level of staining measured on a plate reader, allowing a high throughput comparison of the amount of surface attachment of a number of bacterial strains [30].

The Rm1021 parent strain, the Δ*fliF* mutant and a range of the succinoglycan production mutant strains were grown to late exponential phase and incubated in

microtitre plates without shaking before crystal violet was added to assess the level of irreversible surface attachment by the different strains. The results were however extremely variable. Figure 6-7 shows the results of a typical experiment after 72 hours of static incubation, with error bars showing standard deviation from the mean. Similar results (with similar variability) were obtained for stationary phase cells. It appeared that the *exoS* mutant had enhanced surface attachment compared to the parent strain and other mutant strains, but the variability in the data and between experiments made conclusions from individual experiments difficult. Data was pooled from three repeat experiments to see if there was a consistent difference between surface attachment of the strains. Figure 6-8 shows the results from the pooled data, after 24, 48 and 72 hours (Fig. 6-8A, 6-8B and 6-8C respectively). After 24 hours there does not appear to be a consistent pattern in the difference in surface attachment between the different strains, with all strains showing similar levels of attachment (Fig. 6-8 A). However after 48 hours the *exoS* mutant has higher surface attachment compared to all the other strains (Fig. 6-8B). The same pattern is seen after 72 hours; the *exoS* mutant has a higher amount of staining compared to the other strains, suggesting a greater number of cells attached to the surface (Fig. 6-8C).

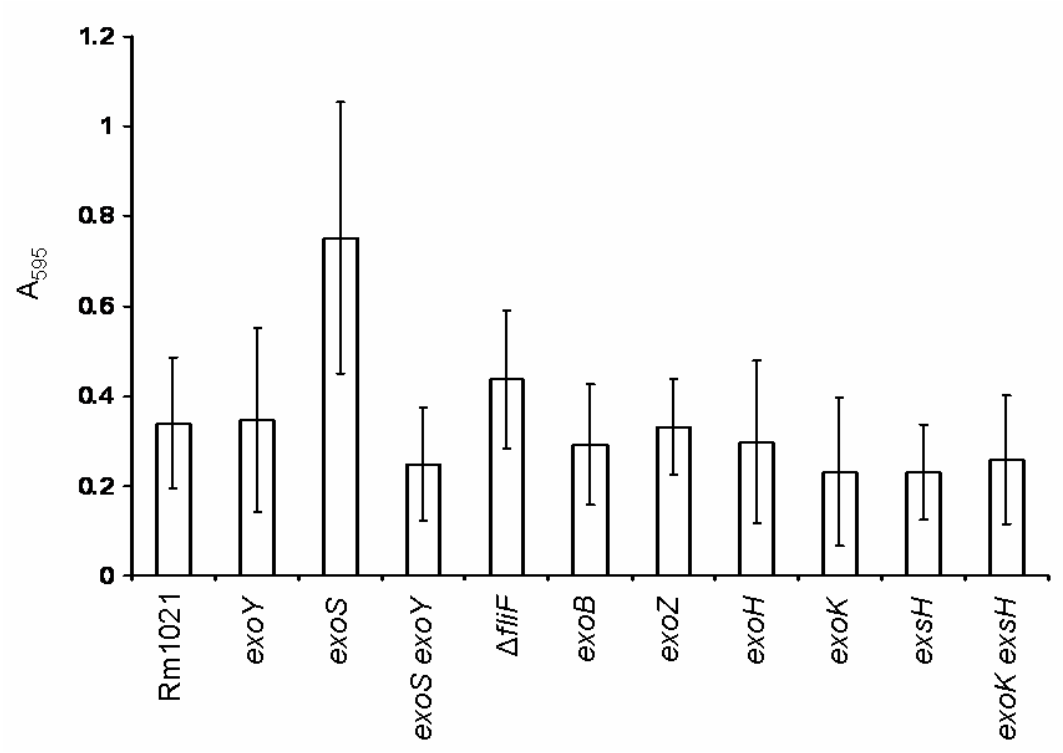


Figure 6-7 Crystal violet staining to assay the extent that the different strains of *S. meliloti* attach to the wells of a polystyrene microtitre plate. Strains were grown to late exponential phase in LB_{MC} and then incubated in microtitre plates for 72 hours without shaking. Error bars are one standard deviations. Repeated experiments gave similar results.

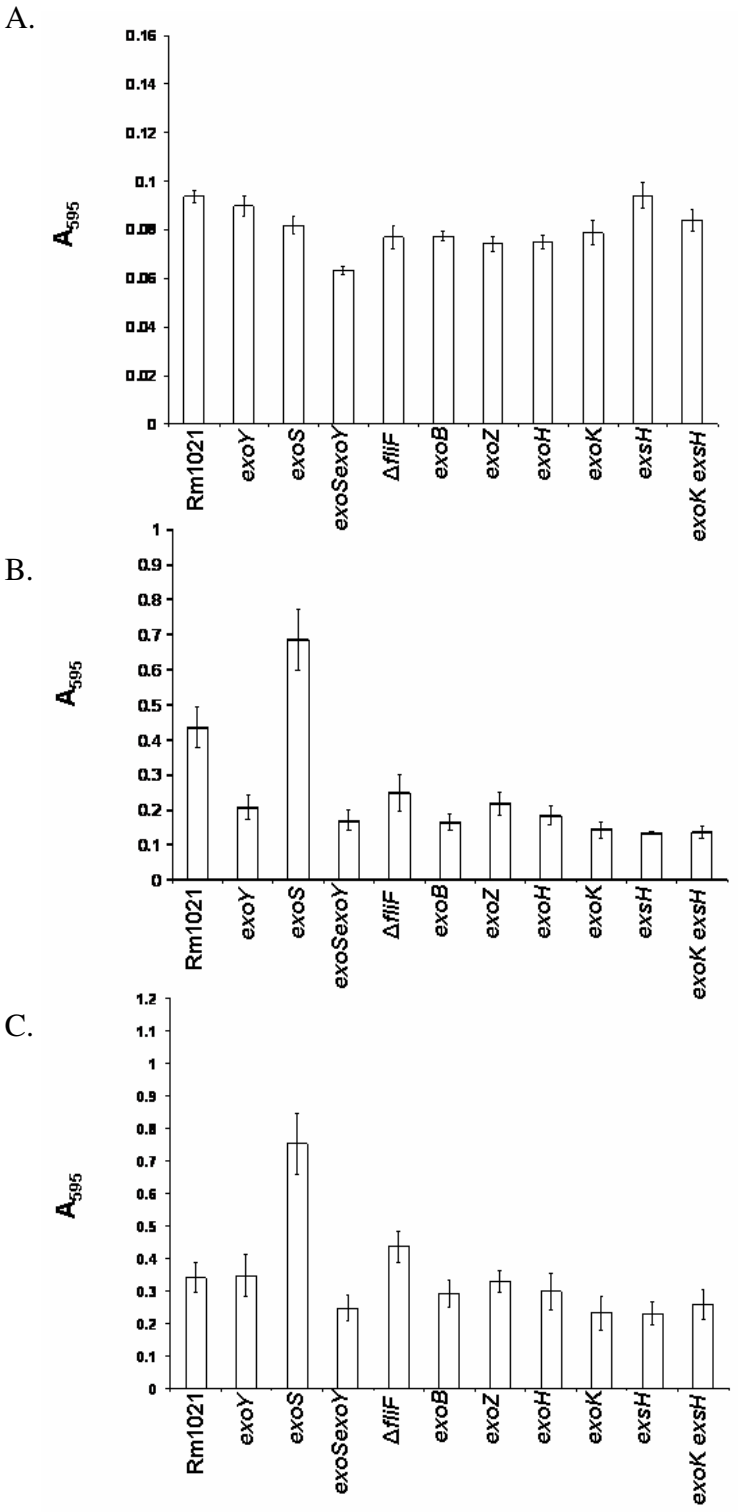


Figure 6-8. Surface attachment over time of *S. meliloti* strains to the wells of polystyrene microtitre plates. Surface attachment was assayed by measurement of the amount of crystal violet staining of attached bacteria after washing the wells. Strains were grown to late exponential phase in LB_{MC} and incubated for A. 24 hours, B. 48 hours and C. 72 hours without shaking at 30℃. Error bars represent standard error.

6.2.2 Evaporation increases the formation of surface-attached biofilms at the air-liquid interface

It therefore appeared that in LB_{MC} the overproduction of succinoglycan by the *exoS* mutant drove the aggregation and sedimentation of the cells by depletion attraction and also leads to a greater number of cells attaching to a surface. At this point however the mechanism of increased attachment by the *exoS* mutant was unclear. One hypothesis was that the increased attachment of the cells was a result of depletion attraction. As well as occurring between the cells, depletion attraction will also occur between the cells and the surface. As such the attractive force generated may be enough to overcome electrostatic repulsion between the cell and the surface resulting in attractive Van der Waals forces allowing the cells to attach. Alternatively the formation of aggregates and resulting sedimentation of those clusters of cells allows the cells to come into transient contact with a surface, again allowing the cells to overcome any electrostatic barrier and form attractive Van der Waals bonds which will facilitate attachment.

Crystal violet staining of the strains showed that the staining was most prominent at the air-liquid interface (Fig 6-9).

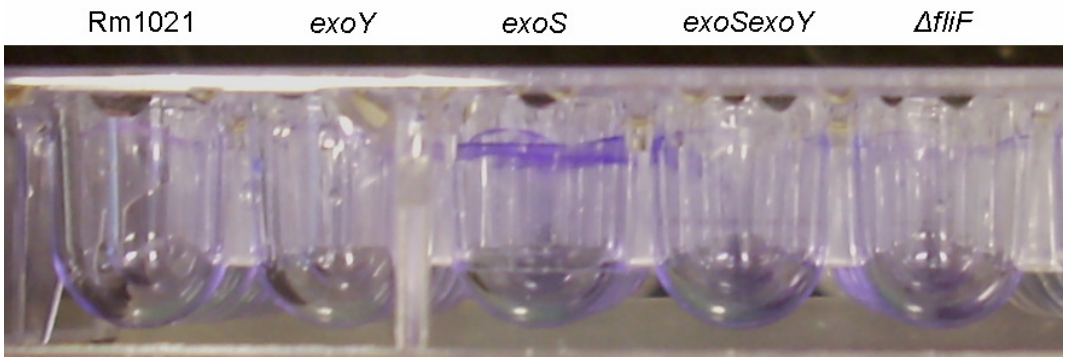


Figure 6-9. Crystal violet staining of bacteria attached to the wells of microtitre plates. The strains were grown to late exponential phase in LB_{MC} and then incubated in the microtitre plates for 72 hours without shaking. After washing the remaining bacteria attached to the surfaces of the wells were stained with crystal violet. The bacteria appear to be attached predominantly at the air-liquid interface.

Due to the static incubation of the wells, aeration of the culture will be poor and as such the greatest growth may occur at the air-liquid interface. This may therefore increase depletion attraction in this relatively oxygenated volume, resulting in greater attachment as outlined above. Alternatively evaporation of the media from the wells at the air-liquid interface could be resulting in deposition of the cells on the sides of the well as evaporation occurred. The microtitre plates for the original screen had been grown in humid Petri dishes but it seemed possible that some evaporation was still occurring. In the case of the *exoS* mutant succinoglycan may also be deposited, which may then further facilitate binding of the cells to the surface and result in increased crystal violet staining.

To test the evaporation hypothesis the strains were inoculated into microtitre plates and incubated without shaking for 72 hours. The plates were incubated in either humid Petri dishes which were regularly supplemented with water, or left under ‘dry’, non-humid conditions which enhanced evaporation. Under non-humid conditions it was found that the amount of staining at the air-liquid interface

increases in all the strains compared to strains grown under humid conditions (Fig 6-10). Within the dry treatment the *exoS* mutant had the greatest staining (Fig. 6-10). Under the humid conditions, where evaporation was minimised, staining was also more apparent in some of the *exoS* mutant wells (Fig. 6-10). Solubilisation of the crystal violet stain with acetic acid and subsequent measurement on a plate reader showed that the strains grown under 'dry' conditions did have a higher level of staining after 72 hours compared to the same strains grown under humid conditions (Fig. 6-11). Comparison of the strains within each treatment showed that the effect of evaporation on surface attachment was greatest in the *exoS* mutant, which had higher staining than the other strains grown under the drier conditions. Under humid conditions it also appeared that the *exoS* mutant had slightly higher attachment than the other strains that were also grown under the humid conditions (Fig. 6-11). Therefore it appeared that enhanced evaporation increased the attachment of cells at the air-liquid interface in all strains, but with the *exoS* mutant having the most prominent staining. However even under conditions where evaporation has been minimised the *exoS* mutant still has enhanced attachment compared to all the other strains.

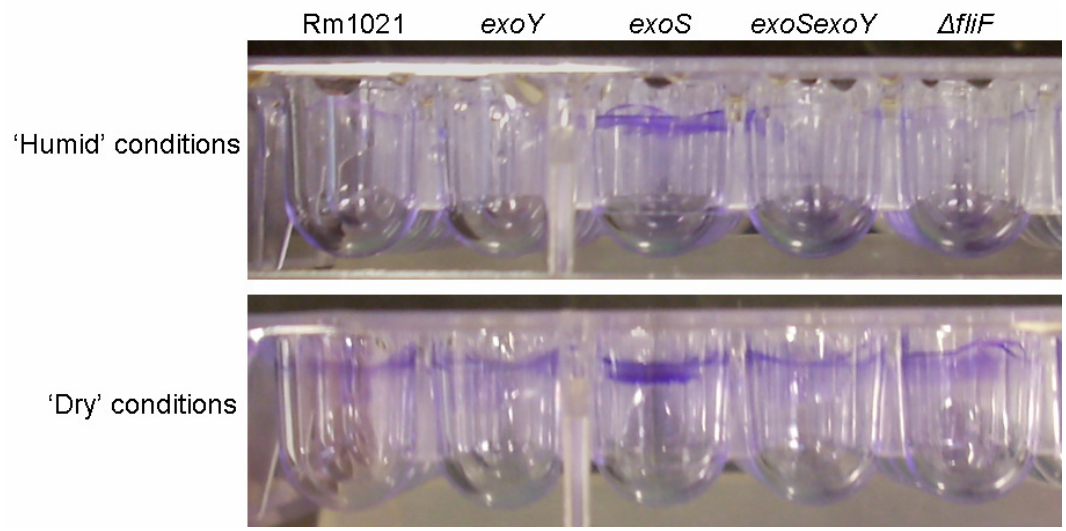


Figure 6-10. Crystal violet staining of bacteria attached to the wells of microtitre plates after 72 hours incubation in a humid Petri dish or under drier conditions which enhances evaporation. Strains were grown to late exponential phase in LB_{MC} and unattached bacteria were washed from the wells. Attached bacteria were then stained with crystal violet.

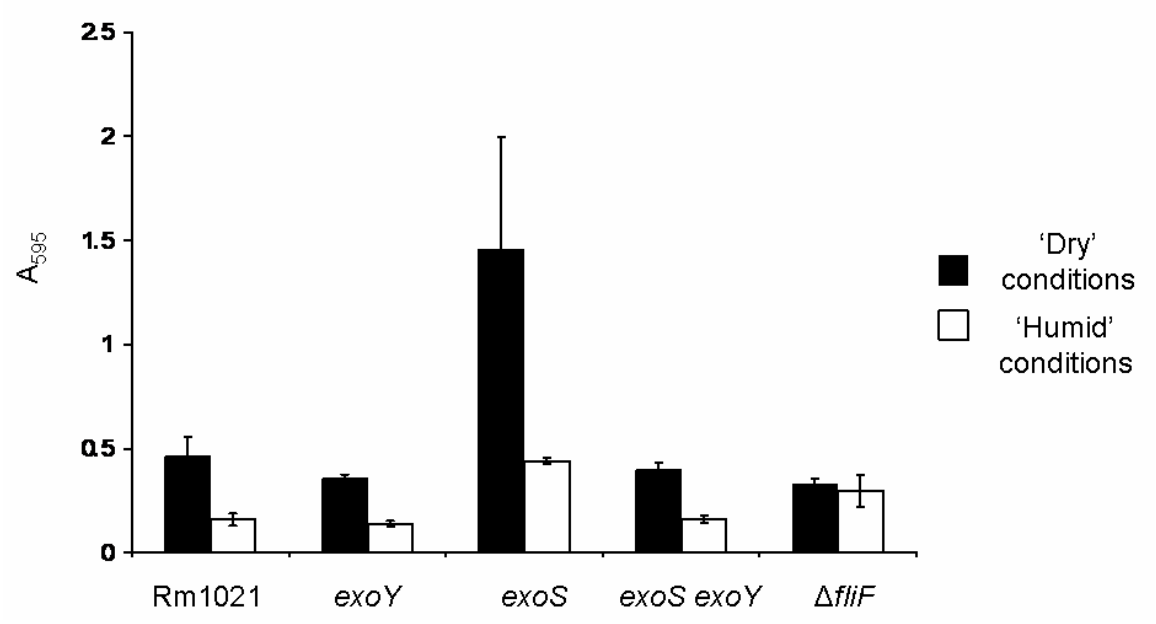


Figure 6-11. Surface attachment of the different strains when grown in a humid Petri dish or outside of the Petri dish, with low humidity enhancing evaporation. Surface attachment was measured by solubilising the crystal violet that stains the attached bacteria and measuring on a plate reader at 595nm. Strains grown to late exponential phase in LB_{MC} and then incubated in microtitre plates for 72 hours without shaking. Error bars represent standard errors.

6.3. The parent strain is also capable of forming surface-associated biofilm structures similar to the *exoS* mutant

In LB_{MC} the *exoS* mutant had therefore been found capable of producing an enhanced biofilm-like structure as a result of the sedimentation of aggregates formed by depletion attraction. It had also been found that the *exoS* mutant, compared to the parent strain Rm1021, had higher surface attachment at the air-liquid interface which was enhanced by evaporation of the media. These results were consistent with what had been seen in previous chapters and suggested that the overproduction of succinoglycan by the *exoS* mutant in LB_{MC} was driving the accumulation of cells on surfaces. However it remained unclear as to whether the parent strain was also capable of similar biofilm formation.

6.3.1 A recently isolated strain of S. meliloti has a similar level of surface attachment as S. meliloti Rm1021

S. meliloti Rm1021 was isolated 26 years ago as a streptomycin derivative of SU47 (which itself had been isolated in 1941), and has been maintained as a laboratory strain [61]. As such the strain is highly domesticated and as a result may have lost certain characteristics seen in a wild type isolate, such as abundant exopolysaccharide biosynthesis. This could also have an effect on surface attached biofilm formation. In *Bacillus* species it has been shown that recently isolated wild type strains form more robust biofilms that have a greater structural complexity than the laboratory strains [157]. It has also been shown that *S. meliloti* Rm1021 is less efficient at the symbiosis than recently isolated strains of *S. meliloti* [158]. To see if Rm1021 was also attenuated for surface-attached biofilm formation it was compared for surface attachment to the wild type isolate *S. meliloti* WSM1022 using the crystal

violet assay, with the microtitre plates incubated without shaking in a humid Petri dish. The wild type isolate *S. meliloti* WSM1022 had a similar level of staining after 72 hours as seen in *S. meliloti* Rm1021, and the Rm1021 *exoY*, *exoSexoY* and Δ*fliF* mutants (Fig. 6-12). The *exoS* mutant has enhanced staining compared to Rm1021 and WSM1022 (Fig. 6-12). The WSM1022 strain also did not appear to form aggregates and remained as a stable culture, the same as Rm1021. This therefore suggests that lack of biofilm formation by Rm1021 is not due to extensive laboratory domestication.

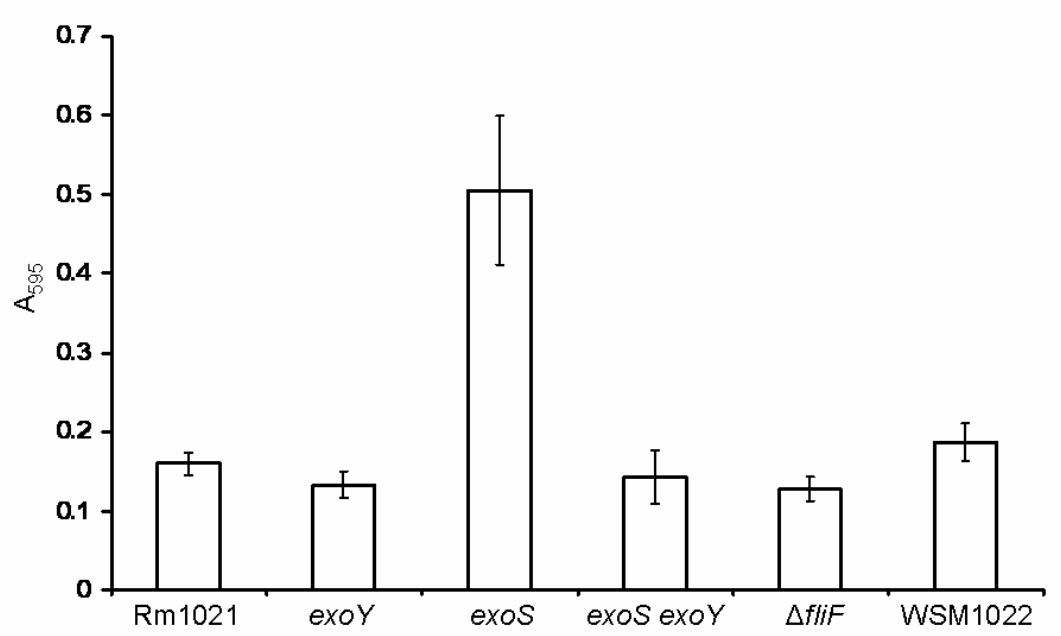


Figure 6-12. Surface attachment of strains of *S. meliloti* Rm1021 compared to a wild type isolate *S. meliloti* WSM 1022. Strains were grown in LBMC for 72 hours and then incubated in microtitre plates without shaking for 72 hours in humid Petri dishes. Microtitre plates were washed and the attached bacteria stained with crystal violet, the level of staining being measured on a plate reader at 595nm after solubilisation by acetic acid. Error bars represent standard errors.

6.3.2 Growth in M9 limited for nitrogen can enhance the formation of a biofilm structure in Rm1021

As the *exoS* mutant has enhanced surface attachment and forms biofilm-like structures similar to other species it was hypothesised that in growth in minimal media that increases succinoglycan production may also lead to greater surface attachment and the development of biofilm structures in the parent strain Rm1021. It has already been shown in the previous chapter that growth of Rm1021 in M9 limited for nitrogen results in enhanced succinoglycan biosynthesis and formation of aggregates with resulting destabilisation of the cultures (Fig. 4-16). Succinoglycan biosynthesis has also been shown to be enhanced in Rm1021 in minimal media limited for phosphate (less than 0.1mM) and with high phosphate (100mM and above) [41]. These amounts of phosphate are thought to be similar to the levels the bacteria will be subjected to in the soil and within the plant cells respectively [41].

S. meliloti Rm1021 was therefore grown in LB_{MC}, minimal modified MOPS with either 0.1mM or 100mM phosphate and M9 and M9 without the addition of NH_4Cl. Biofilm formation was initially screened by confocal microscopy of Rm1021 expressing GFP using the chambered coverslides and imaging the bacteria that had sedimented to the base of the chambers. When grown in M9 limited for nitrogen Rm1021, when incubated without shaking for 24 hours, forms an advanced structure on the base of the chamber, similar to that seen for the *exoS* mutant in LB_{MC} (Fig. 6-13 and Fig. 6-3). The structure appears to consist of highly compacted interconnected aggregates with large spaces in between (Fig. 6-13). Growth in the other minimal media did not appear to enhance the formation of a structure on the base of the

chambered coverslides, and much like as seen in LB_{MC} the cells are subject to Brownian motion (Fig. 6-13).

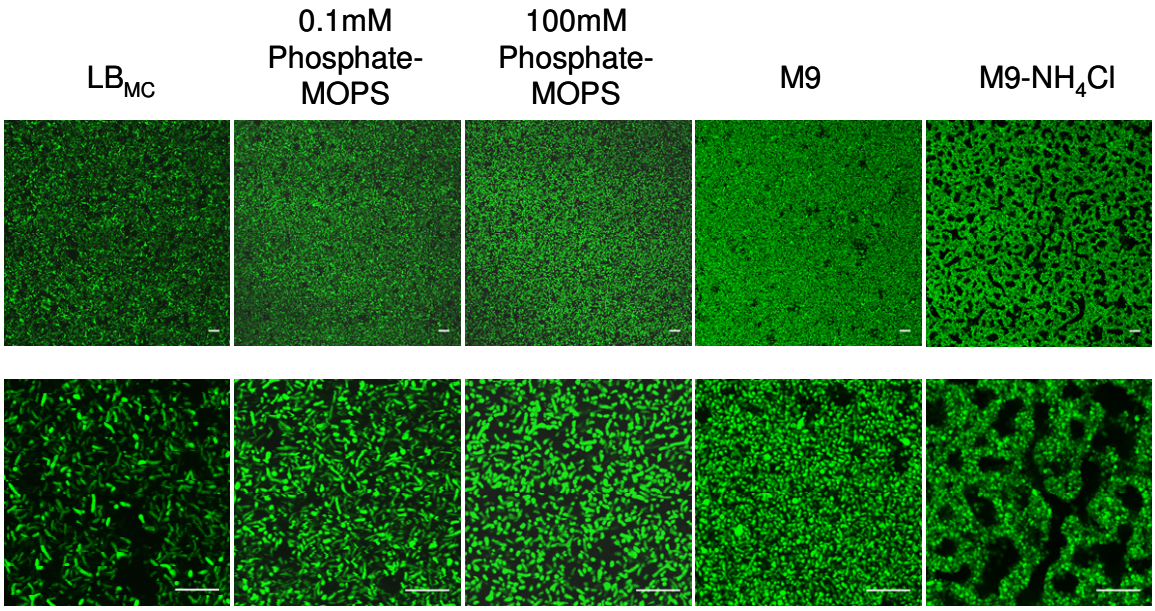


Figure 6-13. Confocal imaging of the sediment formed by Rm1021 expressing GFP when grown in various media. Bacteria were grown in the various media and then put into chambered coverslides. The bacteria that sediment onto the coverslip were then imaged by confocal microscopy after 24 hours incubation without shaking. Bars=10µm.

To show that the formation of this structure by Rm1021 in the nitrogen limited M9 media was due to the increase in succinoglycan, Rm1021 was compared to the succinoglycan biosynthesis mutants *exoY*, *exoS* and *exoSexoY* as well as the Δ*fliF* mutant which is capable of producing succinoglycan at a similar level to the parent strain (Fig. 6-14). This showed that Rm1021, the Δ*fliF* mutant and the *exoS* mutant all formed similar aggregated structures on the base of the coverslides (Fig 6-14). Interestingly it appeared that the Rm1021 and the Δ*fliF* mutant may be forming denser structures than the *exoS* mutant. In contrast to this the *exoY* and *exoSexoY* mutants, which cannot biosynthesise succinoglycan, formed a diffuse mat of cells

and lacked the structure seen in the strains capable of producing succinoglycan (Fig. 6-14).

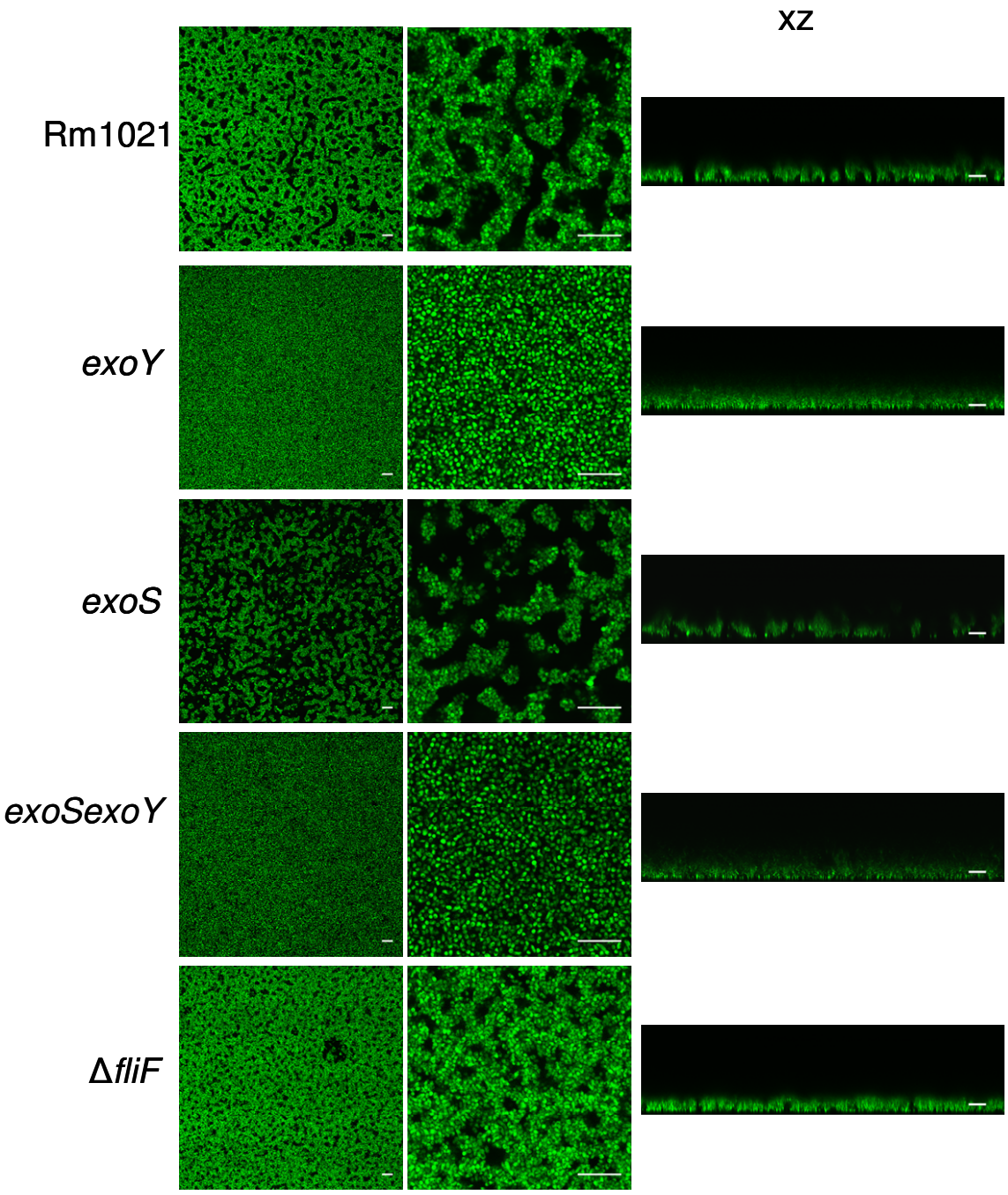


Figure 6-14. Imaging of the sediment formed by *S. meliloti* Rm1021 strains expressing GFP grown in nitrogen limited M9 after 24 hours incubation without shaking in chambered coverslides. Sediment forms on the base of the chamber which is then imaged by inverted confocal microscopy.

6.3.4 Growth in M9 limited for nitrogen results in enhanced attachment of strains that cannot produce succinoglycan

The increased production of succinoglycan in Rm1021 when grown in the nitrogen limited M9 can therefore result in a similar sedimented biofilm structure to the *exoS* mutant. When grown in LB_{MC} the *exoS* mutant also has enhanced surface attachment. To test whether this was also the case for Rm1021 in nitrogen limited M9, surface attachment was measured using the crystal violet assay. Rm1021 was compared to the *exoY*, *exoS*, *exoSexoY* and Δ*fliF* mutants to see if levels of succinoglycan biosynthesis and presence of flagella also affected the amount of surface attachment. Plates were incubated without shaking in humid Petri dishes. Surprisingly after 24, 48 and 72h it appeared that the strains capable of producing succinoglycan (Rm1021, the *exoS* mutant and the Δ*fliF* mutant), were all inhibited in surface attachment (Fig. 6-15A-C). In contrast the *exoY* and *exoSexoY* mutants had a higher level of crystal violet staining than the other strains, although the *exoY* mutant did appear to have greater surface attachment than the *exoSexoY* mutant as well (Fig 6-15A-C). The pattern of staining was also different to what had been seen in LB_{MC} with little staining occurring at the air-liquid interface (Fig. 6-16). The staining covered the entire well, especially at the bottom (Fig. 6-16).

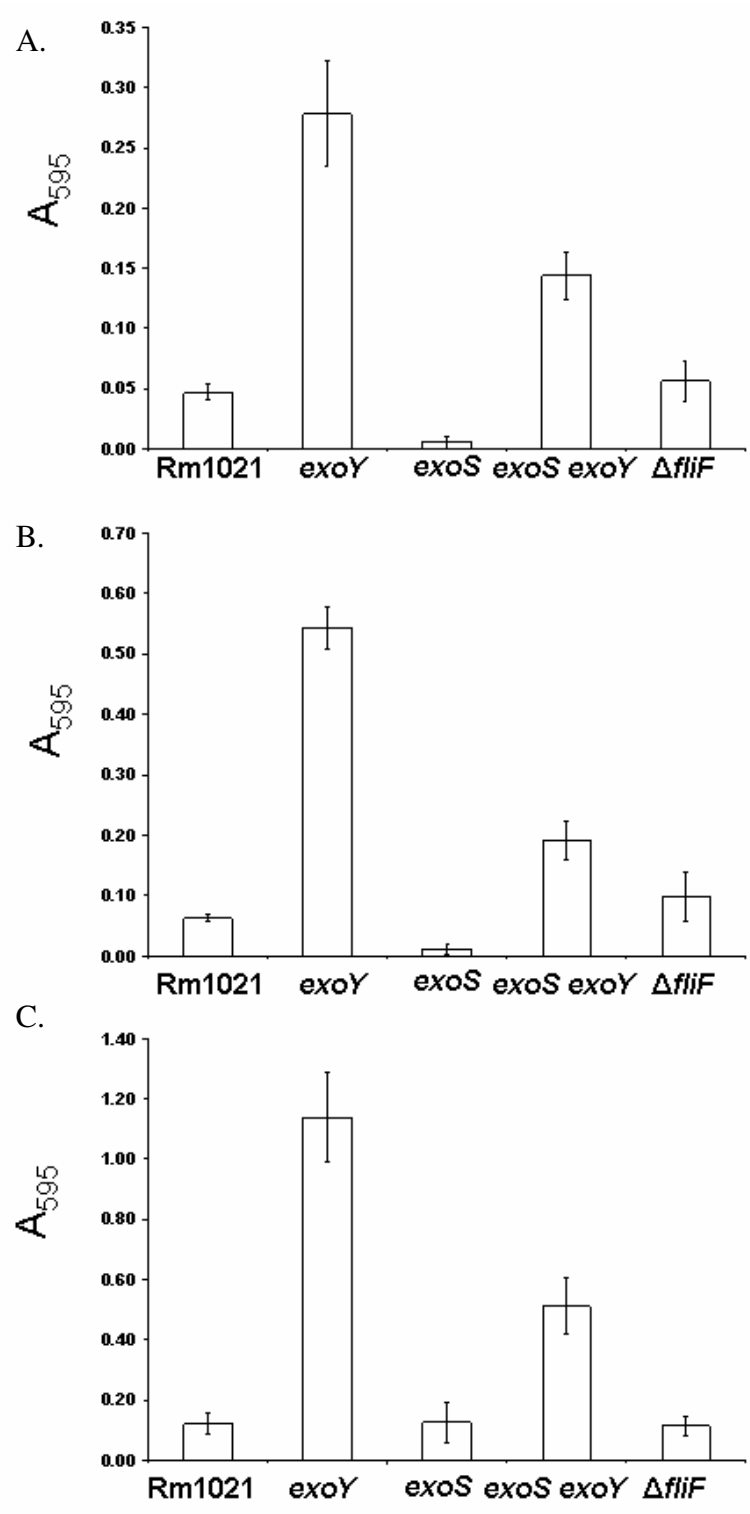


Figure 6-15. Surface attachment of the *S. meliloti* strains grown in nitrogen limited M9. Surface attachment was measured on a plate reader at 595nm after solubilising the crystal violet stained attached cells. Microtitre plates were grown in humid Petri dishes for A. 24 hours, B. 48 hours and C. 72 hours without shaking. Error bars represent standard errors.

A.

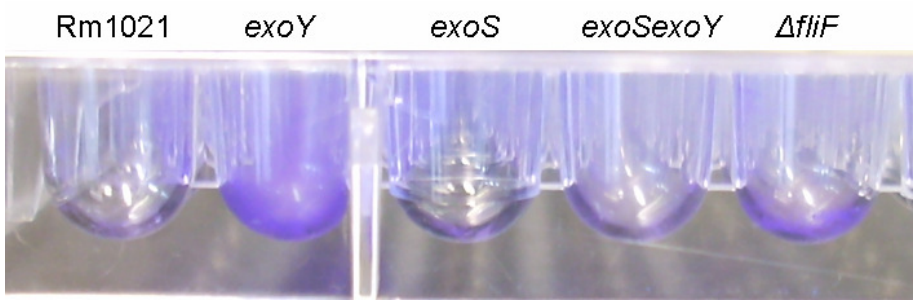


Figure 6-16. Microtitre wells showing the crystal violet staining of the bacteria attached to the surface of the well. Bacteria were grown in nitrogen limited M9 and then incubated in microtitre plates without shaking for 72 hours in humid Petri dishes.

In light of these surprising results the confocal microscopy images were reanalysed. While the *exoY* and *exoSexoY* mutants form a diffuse mat of non-aggregated cells it also appeared from the xy views that the images had a 'speckled' appearance, that could be the result of all the cells being attached to the coverslip on their poles (Fig.6-14). To confirm this hypothesis a z stack of xy images was taken to construct a 3D image of the cells. Figure 6-17 shows the *exoY* mutant expressing GFP after growth in nitrogen limited M9 and after 24 hours of non-shaking incubation. The image shows that all of the cells appear to be located with one of their poles in contact with the coverslip (Fig. 6-17). This therefore suggested when grown in nitrogen limited M9 the *exoY* mutants have enhanced surface attachment and the cells appear to attach to the surface via their poles. A crystal violet assay was also carried out on another succinoglycan biosynthesis mutant *exoB*. This mutant is incapable of synthesising the UDP-galactose that is the activated precursor for succinoglycan biosynthesis and is also affected in LPS synthesis [80]. Growth and static incubation in nitrogen limited M9 showed that the *exoB* mutant also had enhanced surface attachment compared to strains capable of biosynthesising

succinoglycan but was not attached to the same degree as the *exoY* mutant (Fig. 6-18).

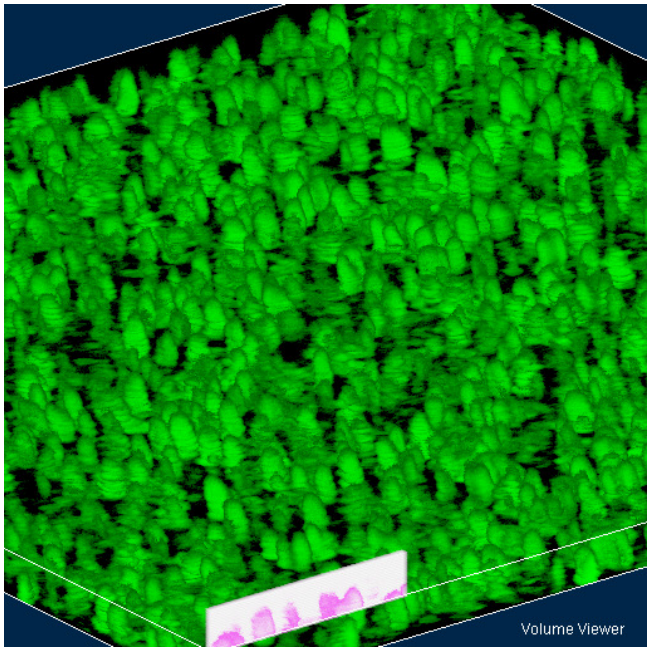


Figure 6-17. 3D representation of *exoY* cells attached to the coverslip by their poles at the base of a chambered coverslide. A z stack of xy images of the *exoY* mutant expressing GFP was imaged to create the 3D representation. Cells were grown in nitrogen limited M9 and then incubated in the chambered coverslides for 24 hours without shaking before imaging. Bar=10µm.

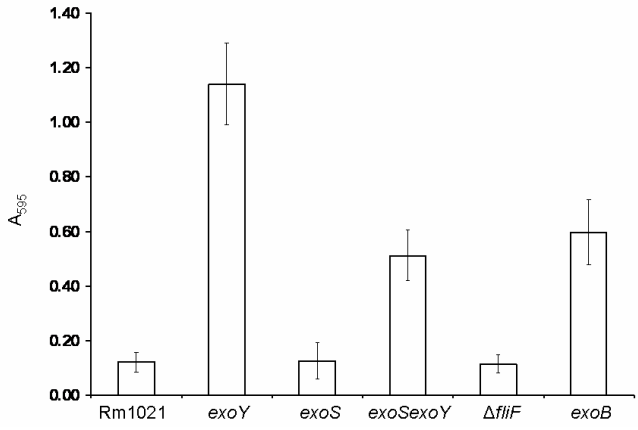


Figure 6-18. Surface attachment of the *S. meliloti* strains grown in nitrogen limited M9. Surface attachment was measured on a plate reader at 595nm after solubilising the crystal violet stained attached cells. Microtitre plates were grown in humid Petri dishes for 72 hours without shaking. Error bars represent standard errors.

The effects of evaporation of the media when the cells are grown in nitrogen limited M9 on surface attachment was also tested via the crystal violet assay. As with LB_{MC} evaporation enhanced surface attachment in all of the strains compared to the humid treatments (Fig. 6-19). This was most pronounced in the *exoS* mutant which had the highest level of attachment under these conditions (Fig 6-19). However despite the enhanced production of succinoglycan by the parent Rm1021 and the Δ*fliF* mutant they had similar levels of attachment to the *exoY* and *exoSexoY* mutants (Fig. 6-19).

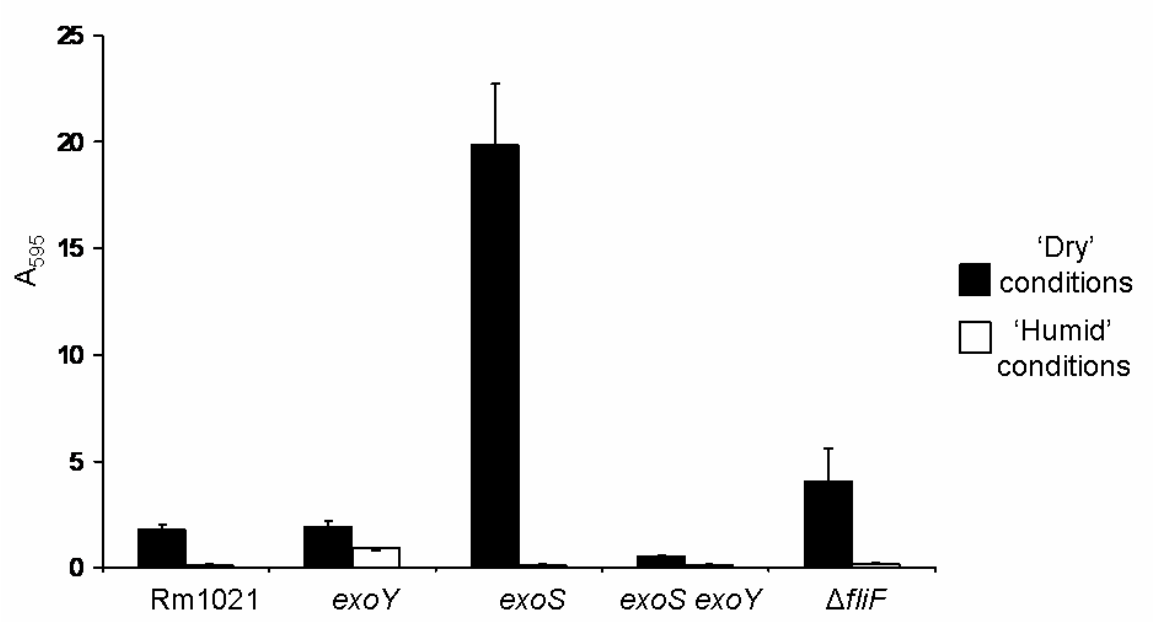


Figure 6-19. Surface attachment of the different strains when grown in a humid Petri dish or outside of the Petri dish with low humidity enhancing evaporation. Surface attachment was measured by solubilising the crystal violet that stains the attached bacteria and measuring on a plate reader at 595nm. Strains grown in nitrogen limited minimal media and then incubated in microtitre plates for 72 hours without shaking. Error bars represent standard errors.

In summary, these results give a complex picture of the role of succinoglycan in surface attached biofilm formation of *S. meliloti*. It appears that the

overproduction of succinoglycan, either via mutation or growth of the cells in minimal media can result in the formation of aggregates, the sediment of which resembles the biofilms formed by other species (Fig. 6-3 and Fig. 6-13 and 6-14). Under evaporative conditions the deposition of cells on a surface results in increased attachment, which is enhanced in the *exoS* mutant (Fig. 6- 11 and Fig. 6-19). Growth in minimal media does however complicate this analysis. It appears that overproduction of succinoglycan in LB_{MC} by the *exoS* mutant enhances surface attachment relative to the other strains (Fig. 6-8C). However in minimal media, mutants unable to produce succinoglycan have greater attachment relative to those strains that can produce succinoglycan (Fig 6-15C). Further it appears that these mutants may be attaching to the surface via their poles, which is not occurring in strains that produce succinoglycan (Fig 6-14).

6.4. The interaction of *S. meliloti* Rm1021 and succinoglycan and flagella mutants with the host plant *Medicago sativa* (alfalfa)

The role of succinoglycan in the attachment of the strains to abiotic surfaces therefore seemed to be a complex process affected by humidity and media conditions. In its natural environment *S. meliloti* attaches to plant roots. To see how succinoglycan biosynthesis and flagella production affected this attachment cell counts were carried out on host plant roots. It was also of some interest as to how these mutants that differed in aggregation and surface attachment performed in the symbiosis with their host plant *Medicago sativa*.

6.4.1. The exoS *mutant does not have enhanced attachment to plant roots*

To assay the number of cells of the different strains attaching to the roots, cell counts on the root surface of *M. sativa* seedlings after four days of growth were analysed. The results showed that the *exoS* and the *exoY* mutant did not have greater attachment (Fig. 6-20).

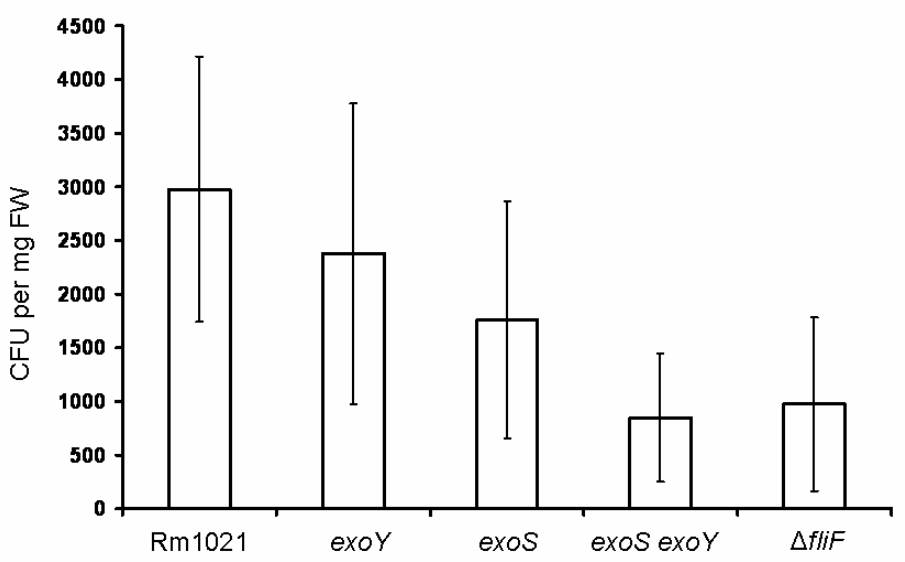


Figure 6-20. Cell counts of bacteria attached to *M. sativa* plant roots 4 days after inoculation. Bacteria were inoculated onto *M. sativa* seedlings which were then incubated at 25°C for 4 days. Roots were excised, weighed, washed and crushed and cell counts performed. Error bars are one standard deviation (n=3 per strain).

6.4.2. The exoS *mutant has reduced virulence compared to the parent strain Rm1021*

The appearance of the nodules formed on the plant roots during a symbiotic event can be used as a measure of the efficiency of a particular strain or mutant. Production of Nod factors by the bacteria in the initial exchange of signals between the bacteria and the plant host are enough to initiate a number of events, including the formation of nodules. However the bacteria may not necessarily then be able to

enter successfully into the root tissue, leaving the developing nodules unoccupied. A successful symbiosis is shown by the formation of pink nodules which is due to the presence of leghaemoglobin, which is synthesised as a way of regulating oxygen levels in the nodule. The production of leghaemoglobin requires the presence of the bacteria; white nodules therefore represent unoccupied nodules and can be a sign that the bacteria may be attenuated in their ability to invade or survive within the plant host. The *exoY* mutant, as it does not produce succinoglycan is unable to initiate infection thread development and so cannot enter the plant nodule; as a result the host plant produces a large number of unoccupied white nodules [123]. The *exoS* mutant is able to successfully enter into a symbiosis but conflicting results have been reported regarding the efficiency of nodule formation and occupation [86, 95]. The formation of pink and white nodules was therefore compared between the different strains. As with previous studies any mutants carrying an *exoY* mutation, that is the *exoY* and *exoSexoY* mutants, are unable to enter the nodules. This resulted in a large number of white nodules, and the plants had stunted growth after 4 weeks (Fig. 6-21 and Table 6-1). In contrast the parent strain Rm1021, the Δ*fliF* mutant and the *exoS* mutant were all able to form pink nodules and fix nitrogen, leading to similar growth of the plants between the bacterial strains (Fig. 6-21). All these strains resulted in the formation of a similar number of nodules on the roots of the plants (Table 6-1). Interestingly however, in contrast to the parent strain Rm1021 and the Δ*fliF* mutant, the *exoS* mutant had a lower percentage of pink nodules (Table 6-1). It therefore appears that the overproduction of succinoglycan by the *exoS* mutant is reducing the efficiency of initiating the symbiosis with its plant host.

Table 6-1. Nodules formed and percentage of total nodules that are pink by different strains of *S. meliloti* Rm1021 on *M. sativa* plants after 4 weeks of growth. Average taken from 5 plants, SE, standard error.

Strain	Total number of nodules ± SE	% of nodules that are pink ± SE
Rm1021	9.8 ± 1.7	89.5 ± 4.5
exoY	25.5 ± 1.7	0 ± 0
exoS	10.2 ± 2.5	51.0 ± 14.1
exoSexoY	15 ± 5.0	0 ± 0
Δ*fliF*	10 ± 1.5	73.6 ± 5.5

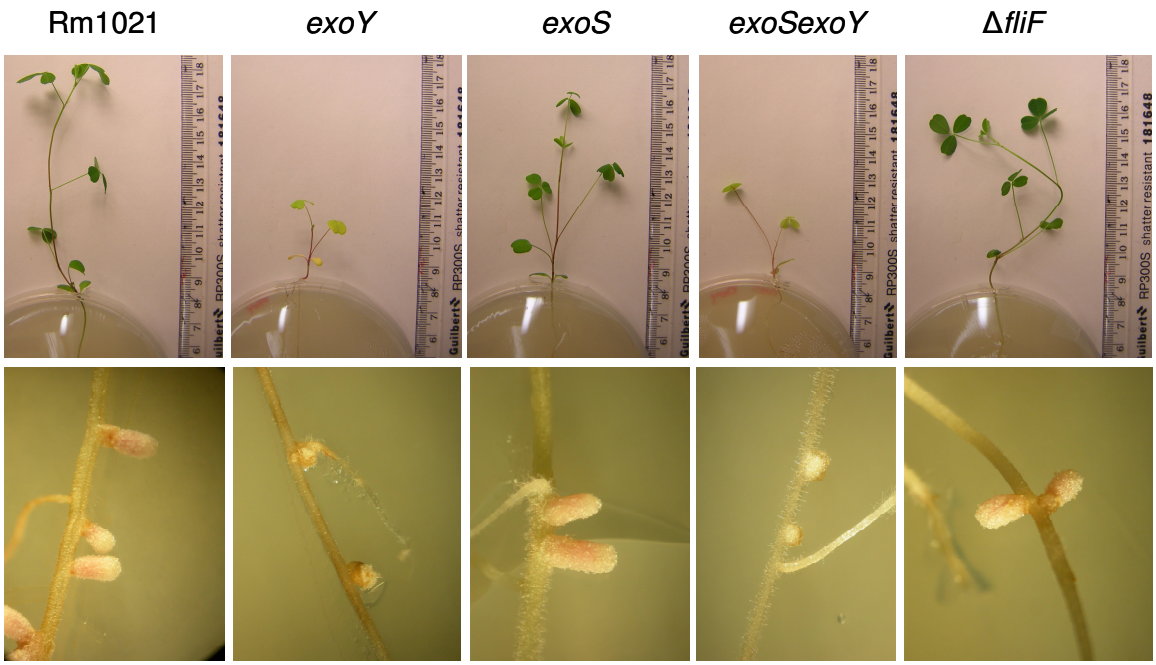


Figure 6-21. Photographs of the plants (top panels) and root nodules (bottom panels) after 4 weeks of plant growth on nitrogen limited media with the plants inoculated with the *S. meliloti* strains as indicated. Images are a representative plant. Rm1021, the *exoS* mutant and Δ*fliF* mutant all form pink nodules allowing normal plant growth. Strains carrying the *exoY* mutation have stunted growth and form only small white nodules on plant roots.

6.5 Discussion

The experiments from the earlier chapters focussed on the formation of cell-cell aggregates of *S. meliloti* Rm1021 and how enhanced production of succinoglycan can drive the formation of these aggregates by depletion attraction *in vitro*. This chapter investigated the formation of surface-associated aggregations, referred to by many authors as biofilms, in *S. meliloti* Rm1021. From these results it is clear that surface-associated biofilm formation in *S. meliloti* Rm1021 is a complex process.

6.5.1. Sedimentation of aggregates formed by depletion attraction leads to the formation of ordered structures that resemble biofilms

In the previous chapters it was shown that the enhanced aggregation of the *exoS* mutant in LB_{MC} was due to the overproduction of succinoglycan by this strain. Succinoglycan causes aggregation by a mechanism of depletion attraction. Further, by growing the parent strain Rm1021 in nitrogen limited M9, which results in an increase in the biosynthesis of succinoglycan, the parent strain also started to form aggregates, which was absent in mutants carrying the *exoY* mutation. Aggregation of the cells results in destabilisation of the cultures and formation of a sediment. When optimising a system with which to image *S. meliloti* biofilms it was therefore interesting to find that previous studies investigating biofilm formation of *R. leguminosarum* and *X. campestris* had imaged these structures using chambered coverslides [22, 98]. As the coverslip forms the base of the culture chamber, microscopy will therefore be imaging the bacteria that have sedimented and are then in contact with the coverslip. The system used in studies of *R. leguminosarum* and *X. campestris* biofilm formation can therefore be used to image the structure of the

sediment formed by the *S. meliloti* cultures. Comparison of the strains showed that the sediment formed by the *exoS* mutant in LB_{MC} had an ordered structure, with laterally aligned cells compacted together, interspersed with large open spaces. Imaging the formation of this structure over time showed that it formed from the accumulation of aggregates on the coverslip. This surprisingly complex structure had many similarities to the biofilms described for other species; the aggregates of cells can be thought of as microcolonies, interspersed with open spaces that could act as water channels. Sedimentation also occurred in the other strains, but due to the lack of aggregate formation in these strains the sediment was a diffuse mat of cells. Imaging of the sediment formed by the different strains in nitrogen limited M9 showed that the parent Rm1021 and the Δ*fliF* mutant, that in this media have increased succinoglycan production which results in aggregation and destabilisation of the cultures, also formed a biofilm-like complex structure similar to that described for the *exoS* mutant in LB_{MC}. This was absent in the strains carrying an *exoY* mutation. It also appeared that in the nitrogen limited M9 the parent Rm1021 and the Δ*fliF* mutant formed denser biofilms than the *exoS* mutant. This supports the observations of the stability of the cultures in nitrogen limited M9 from the previous chapter. It was shown that the level of production of succinoglycan by the *exoS* mutant in this medium results in increased viscosity of the *exoS* cultures which reduces the formation of aggregates and sedimentation. In summary, it therefore appeared that in the presence of succinoglycan depletion attraction was not only driving the formation of aggregates but was also resulting in the formation of ordered structures in *S. meliloti* with similarities to biofilms described for other species.

It was interesting that the structure of the sediment formed by the *exoS* mutant in LB_{MC} and Rm1021 in minimal media was similar to the description of the biofilms formed by *R. leguminosarum* and *X. campestris* using this same system [22, 98]. In particular the biofilm formed by *X. campestris* was described as being composed predominantly of cells attached laterally to other cells and the coverslip, with the production of xanthan essential for formation of an ordered biofilm [22]. As discussed in the previous chapter this lateral arrangement of cells with respect to each other could be indicative of maximisation of the depletion interaction between the cells or between the cells and the surface. It is also interesting that in the previous chapter xanthan was found to be able to aggregate and destabilise *S. meliloti* cultures, consistent with depletion attraction. Aggregation and biofilm formation of *X. campestris* is complex [21, 22]. In rich medium the wild type strain is deficient in aggregate formation but various mutants that are deficient in either production or signal transduction of a signalling molecule that controls xanthan biosynthesis have enhanced aggregate formation [21]. These aggregates were found to be enclosed in a matrix, and xanthan was essential for their formation [21]. However the signalling molecule is also necessary for the release of an extracellular endo-β-1,4-mannanase, which could disrupt the aggregates but does not degrade xanthan [21]. It is therefore possible that an additional polysaccharide is needed for aggregate formation in these conditions [21]. In minimal media different results are found; in this case the wild type has advanced biofilm formation as found by confocal microscopy [22]. In this case no extracellular matrix was reported, and similar to the *exoS* mutant the biofilm formed could be easily disrupted by mixing [22]. This suggests that in minimal media at least

the biofilm formation described by Torres *et al.* [22] could be due to depletion attraction in the presence of xanthan.

6.5.2. Attachment to surfaces by S. meliloti

The biofilm-like structures formed by the aggregation and sedimentation of *S. meliloti* in the presence of succinoglycan were easily dispersed by mixing. This is characteristic of the fact that the aggregates are a result of depletion attraction (see previous chapter). In many bacterial species however surface attached biofilms are resistant to shear forces. The ability of *S. meliloti* to form more permanently attached biofilms was therefore studied by crystal violet staining in polystyrene microtitre plates.

6.5.2.1 Attachment of the strains is affected by the media and evaporation

Crystal violet staining of cells adhering to the surfaces of polystyrene microtitre wells after washing showed that the *exoS* mutant remained attached in greater amounts than the other strains in LB_{MC}. This suggested that the overproduction of succinoglycan by the *exoS* mutant compared to the other strains was facilitating greater attachment.

The mechanism for this increased attachment was unclear. Depletion attraction could be expected to increase not just cell-cell aggregation but also increase cell-surface interactions. However, as shown by the reversibility of the aggregation and sedimentation of the *exoS* mutant, this would not necessarily allow irreversible attachment and may only provide transient surface attachment under low shear conditions. Instead it appeared that evaporation of the medium at the air-liquid

interface was partially responsible for the increased attachment of the *exoS* mutant. The microtitre plates in the initial screen were grown under humid conditions, and the *exoS* mutant appeared to have slightly higher attachment than the other strains. When grown in non-humid conditions which enhanced evaporation, all of the strains had greater attachment to a surface than when grown in humid conditions. However the *exoS* mutant showed the greatest increase in crystal violet staining. It therefore seems possible that the evaporation of the medium results in deposition of the cells and also in the case of the *exoS* mutant succinoglycan, onto the surface of the microtitre well, with the presence of succinoglycan allowing stronger attachment to the surface.

It therefore appears that succinoglycan could be playing two different roles. Under aqueous conditions aggregation and sedimentation of the cells will be the result of depletion attraction. However if exposed to desiccation stress it appears that the deposition of cells and succinoglycan can facilitate greater attachment of the cells to a surface. This could have a selective benefit in the environment, where the cells and plant roots may be exposed to periods of drying. As drying occurs the deposition of the cells and succinoglycan onto the roots of the host plant might prevent the cells from being washed away when wetting of the soil eventually occurs. It is also possible that succinoglycan might directly protect the cells from desiccation stress. It has been hypothesised that because bacterial polysaccharides are hygroscopic, allowing large quantities of water to be bound, they could thus provide a hydrated film to protect bacteria from desiccation [40]. It has been shown in a number of *Pseudomonas* species, which attach to plants either on leaves or roots, that

production of aggregates and a hydrated polysaccharide matrix can increase the survival of the bacteria in response to desiccation stress [159-161].

6.5.2.2 Attachment of a wild type isolate of S. meliloti is not greater than that seen in the Rm1021 strain in LB_{MC}

In chapter three it was found that the parent strain Rm1021 did not appear to be producing succinoglycan, and as a result lacked aggregation. In this chapter it has also been found that the lack of succinoglycan production by this strain in LB_{MC} also means it does not attach to surfaces as well as the *exoS* mutant, as shown by crystal violet staining. This lack of exopolysaccharide production by Rm1021 could be a result of domestication of the strain through successive culturing in the laboratory [38]. During the course of this study it was also found that the Rm1021 strain was a less efficient symbiont on the model host plant *M. truncatula* than recently isolated *S. meliloti* strains [158]. It has been shown in *Bacillus* species that recently isolated strains form biofilms more readily, with more complex structures than strains that have been maintained in the laboratory [157]. It therefore seemed possible that the Rm1021 strain may also be lacking in surface–attached biofilm formation. To find out if this was the case surface attachment on microtitre plates was compared between *S. meliloti* Rm1021 and a recently isolated wild type strain WSM1022. It appears that under these conditions surface attachment between Rm1021 and WSM1022 are similar. It therefore seems that the laboratory strain of *S. meliloti* Rm1021 is not deficient in surface attachment compared to a recently isolated *S. meliloti* strain WSM1022 in LB_{MC}. Further analysis of the strain by microscopy and growth in minimal media may show if there are any differences between Rm1021 and WSM1022 that are not seen in LB_{MC}.

6.5.2.3. Surface attachment of the exoY mutant is enhanced in nitrogen limited M9

Having shown that the Rm1021 strain did not appear to be deficient in surface attachment compared to a wild type isolate it was hypothesised that growth in minimal media may increase the surface attachment and biofilm formation of Rm1021. As had been shown in the aggregation studies in the earlier chapters, growth in nitrogen limited M9 results in enhanced aggregation of Rm1021 due to increased succinoglycan biosynthesis. The resulting sediment also has a biofilm-like structure similar to that seen in the *exoS* mutant in LB_{MC}, but only has a transient attachment to the surface (see above). To see if growth in nitrogen limited M9 also increased surface attachment, as had been seen for the *exoS* mutant in LB_{MC}, crystal violet assays were carried out. Surprisingly in this minimal media the *exoY* mutant, which cannot produce succinoglycan, had greater attachment than any of the strains that could produce succinoglycan. The *exoSexoY* mutant also had greater attachment than any of the strains producing succinoglycan, but less than the *exoY* mutant.

The mechanism of the increased attachment of strains carrying an *exoY* mutation compared to those strains that can biosynthesise succinoglycan is unclear. In contrast to observations in LB_{MC}, the staining occurred over the entire well, not just at the air-liquid interface. Analysis of the confocal images showed that the *exoY* mutants appeared to be attaching to the coverslip via their poles, which was not occurring in the strains capable of producing succinoglycan. This raises the possibility that in nitrogen limited M9 the *S. meliloti* strains are producing a polar adhesin that enhances surface attachment. Polar attachment of cells to a surface has been seen in various species of bacteria, including the nitrogen fixing symbiont *R.*

leguminosarum. In a study of *R. leguminosarum* biofilm formation, confocal microscopy showed that the cells in a chambered coverslide system appeared to attach to the coverslip via the poles of the cell giving the biofilm a 'honeycomb' arrangement [98]. While the structure behind this attachment was unidentified, it was found that mutants defective in biofilm formation were unable to secrete proteins that had N terminal sequences identical to predicted proteins encoded by *rap* genes [98]. The *rap* genes were identified as encoding proteins secreted by the cells, one of the domains of which bound to the polar regions of *R. leguminosarum* cells, possibly to some component of the outer membrane or capsular polysaccharide [98, 162]. Also *R. leguminosarum* cells are known to produce a polysaccharide at the poles of the cells called glucomannan, which is essential for attachment to roots at acidic pH [163]. A gene required for the synthesis of glucomannan *gmsA* was found to be conserved in *Rhizobia*, with a homologue also found in *S. meliloti* [164]. However in this case attachment to the root surface is thought to be mediated by a host plant lectin [163]. Polar adhesion is also seen in *Caulobacter* species which produce a polar 'holdfast' that facilitates attachment to a surface and is also composed of carbohydrates [165]. It therefore appears that the production of polar adhesins is widespread and could also be the case in the *S. meliloti* Rm1021 *exoY* mutant under the correct conditions. It is interesting that the production of succinoglycan appears to prevent polar binding of the cells. It could be that the depletion attraction as a result of succinoglycan prevents the cells arranging themselves for polar adhesion. Alternatively if the cells are producing a polar adhesin it could be composed of polysaccharides on the polyprenyl lipid membrane carriers that also make succinoglycan. The production of

succinoglycan in Rm1021 may therefore prevent the construction of this polysaccharide in all cells not carrying the *exoY* mutation.

Under humid conditions it appeared that not only did the *exoY* mutant have enhanced attachment to the other strains but that the *exoS* mutant may actually be inhibited in surface attachment. This may again be a result of the viscosity of the medium as a result of the upregulation of succinoglycan biosynthesis, which appears to stabilise the *exoS* mutant when grown in nitrogen limited M9 (see previous chapter and above).

Finally, as was found with the LB_{MC} treatments, growth in dry condition enhanced evaporation and surface attachment of all strains (see above). As was found in LB_{MC} overproduction of succinoglycan by the *exoS* mutant massively enhances surface attachment. It would perhaps be expected therefore that under evaporative conditions the parent strain Rm1021 would have greater surface attachment that mutants carrying the *exoY* mutation due to the upregulation of succinoglycan biosynthesis by Rm1021 in nitrogen limited M9. This did not appear to be the case with equal staining between the wells of the parent Rm1021 and the *exoY* mutant. However the increase in staining for the parent Rm1021 was far greater, as the *exoY* mutant already had so many cells attaching to the wells.

In summary it appears that surface attachment of *S. meliloti* Rm1021 is complex and is affected by succinoglycan production, desiccation and the medium in which the cells are grown. The surface attachment of other legume symbionts has been relatively well studied and further work will be needed to elucidate if there are similar factors at play in *S. meliloti* Rm1021.

6.5.2.4 Attachment of the strains to plant roots and relationship to virulence

Having shown the altered aggregation and surface attachment of the *exoS* mutant *in vitro* the interaction of the strain with the host plant was investigated. Surface attachment on roots was measured by cell counts on *M. sativa* seedlings after one week incubation. Perhaps due to the complexity of the root environment the results were variable and it was difficult to draw any conclusions with all of the strains showing a similar level of attachment.

The virulence of the strains with the host plant was also measured by inspecting plant growth and nodule number and colour on plants grown on nitrogen limited media. This has previously been investigated for the *exoY* and *exoS* strains used in this study [60, 86, 131] although there is some disagreement on the effect of the *exoS* mutation on virulence [86, 95]. As had been found previously the strains carrying the *exoY* mutation were completely unable to enter into the symbiosis; this is because succinoglycan acts as a signal for the initiation of the formation of infection threads [60, 123, 131]. This gives rise to a large number of white nodules on the roots, and stunted, poor growth of the plants. Plants incubated with the parent strain Rm1021, Δ*fliF* mutant and *exoS* mutant all had a similar number of nodules. However the *exoS* mutant had a lower percentage of occupied, pink nodules (~50%) than the parent strain (~89%) or Δ*fliF* mutant (~73%). This was in agreement with Wells et al. (2007) who had found that the *exoS* mutant has lower virulence than the parent strain [95].

The reason for this reduced virulence is unclear. It was suggested from studies of infection thread formation using GFP expressing bacteria that the *exoS*

mutant has reduced efficiency in colonisation of curled root hairs [123]. The Δ*fliF* mutant has only slightly fewer pink nodules than the parent strain and more than the *exoS* mutant. This would suggest that the loss of motility in the *exoS* mutant is possibly playing a minor role in the reduced virulence of this strain. Like this study it has been shown previously that motility is not essential for nitrogen fixation [95].

An alternative hypothesis is that the enhanced aggregation or surface attachment of the *exoS* mutant means it is unable to enter into the symbiosis as readily as the other strains. A similar hypothesis was proposed by Wells *et al.* who also found that the *exoS* mutant has enhanced surface attachment in LB_{MC} with crystal violet assay and proposed that the mutant was 'locked' in a biofilm state [95]. It has been shown from studies on *P. aeruginosa* biofilms that mutants which have enhanced surface attachment or cell-cell aggregation also have reduced virulence [166, 167]. These results are of particular relevance to this study as the *P. aeruginosa* mutant resulted in inactivation of the *retS* gene, which encodes a two component regulator [166]. It has therefore been suggested that bacteria need to modulate a trade-off between an aggregative biofilm mode of growth and a single-celled state which allows them to infect a host [167]. Further to this two component regulators may be a pivotal part of the pathway which acts to alter cell responses as a result of environmental stimuli and may therefore be the point of control that the cell uses to modulate the trade-off between aggregation and biofilm formation or virulence [167]. It is therefore possible that the two component regulator ExoS may be playing a similar role in *S. meliloti*.

Chapter 7

Concluding remarks

The production of extracellular polysaccharide is integral to the process of aggregation in many bacteria. In this study the physicochemical mechanism underlying bacterial aggregation in the presence of polymers has been investigated. It has been shown that succinoglycan, a polysaccharide produced by *S. meliloti* Rm1021, is capable of causing aggregation of the cells via a mechanism of depletion attraction.

The initial aim of this project was to find out whether production of succinoglycan could result in aggregation of *S. meliloti* Rm1021. The biosynthesis of the exopolysaccharide succinoglycan has been intensively studied in *S. meliloti*. The *exo* genes and the role of their products in the steps of the succinoglycan biosynthetic pathway have been extensively characterised [77]. Screening succinoglycan biosynthesis mutants grown in LB_{MC} isolated an *exoS* mutant that had enhanced formation of cell aggregates, which then sediment to the bottom of the culture vessel. ExoS is the sensor kinase of a two component regulatory system in *S. meliloti* that regulates the transcription of succinoglycan biosynthesis genes and genes responsible for flagella synthesis [92, 93]. The *exoS* mutant is a gain of function mutant that results in overproduction of succinoglycan and downregulation of flagella synthesis [92, 93]. Addition of succinoglycan isolated from the *exoS* mutant resulted in aggregation of all of the *S. meliloti* strains tested. The aggregative phenotype of the *exoS* mutant is the result of overproduction of succinoglycan compared to the parent strain Rm1021.

When grown in LB_{MC} the parent strain Rm1021 does not appear to produce high enough levels of succinoglycan to result in aggregation. However growing *S. meliloti* Rm1021 in nitrogen limited minimal media, which causes upregulation of the biosynthesis of succinoglycan, also resulted in aggregation of the cells. Measurement of the molecular weight of purified succinoglycan showed that the addition of the HMW polymer to cultures was responsible for aggregation of the cells. The LMW form of succinoglycan has been previously demonstrated to have a signalling role in infection thread formation in the initiation of the symbiosis between the bacteria and the plant host [123]. This is the first time that the HMW form of the polymer has been shown to have a role in the biology of *S. meliloti* Rm1021.

While the role of extracellular polysaccharides in bacterial aggregation is well documented, the mechanism by which the polymers result in aggregation of the cells is less understood. Most studies of aggregation or biofilm formation assign a bridging role to the polymers, whereby the polysaccharide interacts with the surfaces of multiple cells, drawing them together into a floc or aggregate. This results in an extracellular matrix, the cells encased in a layer of polysaccharide. In *S. meliloti* Rm1021 this is not the case. Microscopy of the aggregates showed that the cells were not encased in extracellular polysaccharide and instead the polymer is dispersed throughout the medium. It therefore appeared that another mechanism of aggregation was occurring. Aggregation by polymer in colloidal dispersions can occur via two main mechanisms: bridging flocculation or depletion attraction. Depletion attraction is a crowding phenomenon (and is referred to as macromolecular crowding in the biological literature), and is based on the maximisation of the entropy of a colloidal system in the presence of non-adsorbing polymers [105]. The fact that the polymers

cannot interpenetrate the particles decreases the osmotic pressure around the particles, so that when they come closer together solvent diffuses out of the gap between them, pulling the particles together. The decrease in the surface area of the particles that is exposed to the solvent and the polymer means that the polymer then has access to a greater volume resulting in an increase in entropy of the polymer and the system as a whole [105]. For the colloidal particles the aggregation results in destabilisation of the dispersion due to the sedimentation of the aggregates. In the case of depletion attraction this phase separation means that the polymer is then concentrated in the upper, cleared phase.

Aggregation of *S. meliloti* by succinoglycan appears to occur via depletion attraction rather than bridging flocculation. There are several lines of evidence to support this. First, as mentioned above, there does not appear to be any succinoglycan adsorbing to the cells or the aggregates formed by the cells in the presence of succinoglycan. This is shown by microscopy but is also supported by the fact that both the cells and the polysaccharide carry a net negative charge which would lead to electrostatic repulsion. Adsorption of the polysaccharide to the surface of the cells could also lead to an unfavourable decrease in entropy. The arrangement of the cells within the aggregates also favours the depletion attraction hypothesis. The strength of the attraction between the particles is determined by the osmotic pressure ($\prod$, due to the polymer not adsorbing to the surface of the particle) $\times$ $V_{overlap}$ between the particles [107]. As the bacterial cells can be thought of as cylinders the most stable interaction between the cells will occur if the cells align laterally as this will give the greatest $V_{overlap}$. Again, as seen by microscopy, all of the aggregates formed by *S. meliloti* are composed of laterally aligned cells, which would increase

the depletion attraction. It is also possible that the conformation of succinoglycan favours depletion attraction. Succinoglycan has been shown to adopt a stiff rod-like conformation [73, 138, 140]. Rods have been shown to increase the strength of the depletion interaction between colloidal particles [141-143]. This is due to the greater rotational degrees of freedom that the rods occupy, leading to a greater increase in entropy when the particles aggregate [141-143]. Finally, the aggregation and destabilisation of *S. meliloti* by succinoglycan is a crowding phenomenon. Experiments which tested the dependence of the stability of the cultures on cell and succinoglycan concentration found that the limiting succinoglycan concentration for destabilisation decreased with increasing cell concentration. This is in contrast to what would be expected for bridging flocculation, where a greater concentration of cells would correspondingly require a greater concentration of polymer for aggregation to occur [116].

Depletion attraction may be of widespread importance in bacterial aggregation and ecology. Addition of succinoglycan also resulted in aggregation and destabilisation of stationary phase and non-flagellated mutant *E. coli* MG1655 cultures and polystyrene colloidal particles. This raises the possibility that in the multispecies environment of the rhizosphere the production of succinoglycan could also result in aggregation of other species by depletion attraction. It could also result in aggregation of colloidal matter, such as soil particles. Analysis of the effect of other polymers on *S. meliloti* cultures showed that xanthan is also capable of causing aggregation via depletion attraction. Overall therefore, this is the first time that a bacterial polysaccharide has been shown to cause aggregation, not just in the bacteria that produce that polysaccharide but in other bacterial species and colloidal systems.

Further to this, addition of xanthan to *S. meliloti* cultures resulted in destabilisation consistent with depletion attraction. At least one other bacterial exopolysaccharide can therefore aggregate bacteria via depletion attraction. This supports the notion that depletion attraction by bacterial polysaccharides could be a more ubiquitous phenomenon than currently appreciated.

The role of flagella in the aggregation of bacteria by depletion attraction remains unclear. It became apparent that compared to *E. coli* MG1655, *S. meliloti* Rm1021 is less motile and electron microscopy showed that many of the cells lacked flagella. In the case of motile late exponential phase cultures of *E. coli* MG1655, addition of succinoglycan did not result in aggregation or destabilisation of the culture as had been seen in *S. meliloti*. Mutants lacking flagella or stationary phase cultures of *E. coli* MG1655 (when flagella synthesis and motility is downregulated [150]), do aggregate and the cultures destabilise in the presence of succinoglycan. In *E. coli* MG1655 flagella therefore appear to have an inhibitory effect on aggregation by succinoglycan. Addition of succinoglycan to *S. meliloti* Rm1021 resulted in aggregation at all growth stages. This difference between the two species could be due to the fact that *S. meliloti* Rm1021 is relatively lacking in flagella and motility. Observation by microscopy showed that *S. meliloti* Rm1021 cells in the presence of succinoglycan that were actively motile were not in aggregates, which suggests that motility enables the cells to overcome the depletion attraction. It is thought that in many bacteria the switch to an aggregative phenotype is accompanied by a reduction in motility along with an increase in the production of extracellular polysaccharides [155]. A similar alteration in motility and exopolysaccharide production may therefore be occurring to allow bacterial aggregation via depletion attraction. Further work will

be required to elucidate whether the presence of flagella or actual motility is needed for cells to overcome depletion attraction.

Studies of bacterial aggregation, as well as looking at cell-cell aggregation also focus on surface attached aggregates which are often referred to as biofilms. Many bacterial aggregates are found associated with a surface, which may then allow them to occupy and persist in a favourable niche. In the case of *S. meliloti* in the presence of succinoglycan, association with a surface occurs after sedimentation of the aggregates. Interestingly previous studies of surface associated aggregates of the plant-associated bacteria *R. leguminosarum* and *X. campestris* have utilised imaging systems that allow the visualisation of bacteria that settle onto a coverslip. This same system was used to visualise the sediment formed by the *S. meliloti exoS* mutant. The structure of the sediment was surprisingly complex and carried many of the hallmark features associated with the surface-associated biofilms studied by microbiologists in other species. This included the formation of interlinked aggregates or 'microcolonies' interspersed with large gaps or 'water channels'. This enhanced biofilm-like structure was also seen in the parent strain Rm1021 when grown in minimal media that enhances succinoglycan production, but was absent in mutants unable to synthesise succinoglycan. The formation of depletion-induced aggregates can therefore also lead to the development of an enhanced biofilm-like structure associated with a surface. Unlike some other biofilms however this structure was not firmly attached to the surface and mixing or shear forces could disrupt the structure.

Other processes can modulate surface attachment of *S. meliloti* Rm1021. Evaporation of the medium appears to result in deposition of the cells onto a surface facilitating stronger attachment, which is enhanced by increased succinoglycan

production in the *exoS* mutant. It also appears that under minimal media conditions the cells may be able to produce a polar adhesin, which facilitates stronger attachment in mutants that cannot produce succinoglycan. In this case aggregation of the cells by depletion attraction may prevent the cells from orientating their poles to the surface. However, assaying the number of bacteria attached to the roots of host plants showed no difference between the parent strain and the succinoglycan biosynthesis mutants. It was also interesting to note the difference in virulence between the aggregative *exoS* mutant and the parent strain Rm1021. While it is possible that another phenotype caused by the *exoS* mutation could impact on the symbiosis it is tempting to speculate that the enhanced aggregation by depletion attraction may prevent the cells from entering into the symbiosis as readily as the non-aggregating parent strain.

From these results it is possible to suggest a model of how succinoglycan-driven depletion attraction and environmental factors may modulate aggregation and surface attachment of *S. meliloti* Rm1021 with respect to its plant host. First, from this study it appears that the ExoS two component regulator plays a central role in modulating aggregation via production of succinoglycan. The level of succinoglycan production is critical to aggregation by depletion attraction and the degree of surface attachment under evaporative conditions. The ExoS sensor may therefore coordinate the cells' production of succinoglycan and thus aggregative ability in response to environmental conditions.

A more general conclusion of how aggregation of *S. meliloti* may affect the symbiosis with the plant host can also be hypothesised (Fig. 6-1). As mentioned previously the rhizosphere is likely to be a complex heterogeneous environment,

composed of multiple species in a number of microniches. Under these minimal conditions it appears likely that *S. meliloti* Rm1021 may have upregulated biosynthesis of succinoglycan, which could result in the accumulation of the polysaccharide and depletion attraction both between the cells and between the cells and the surface of the root. This could facilitate niche occupation by allowing the cells to form stable aggregates and biofilm-like structures near to or on the host root (Fig. 6-1). If there were bouts of drought, evaporation of the water from the microniche may lead to deposition of cells and succinoglycan, allowing stronger attachment of the cells and possibly preventing them from being dislodged when the drought ends. Finally the fact that the depletion attraction is reversible and that actively motile *S. meliloti* Rm1021 cells did not aggregate in the presence of succinoglycan could be a selective benefit with respect to the initiation of the symbiosis. When the plant releases the signals for the initiation of the symbiosis the bacteria may upregulate flagella production allowing them to overcome the depletion attractive force between the cells and move towards the root hair. In the biofilms formed by other species it is often thought that the presence of an extracellular matrix may actually 'trap' the cells [155]. In the case of aggregation by depletion attraction this is not the case and may be a selective benefit for *S. meliloti* by allowing the cells more flexibility between aggregation when free-living and entry into the symbiosis with the plant host.

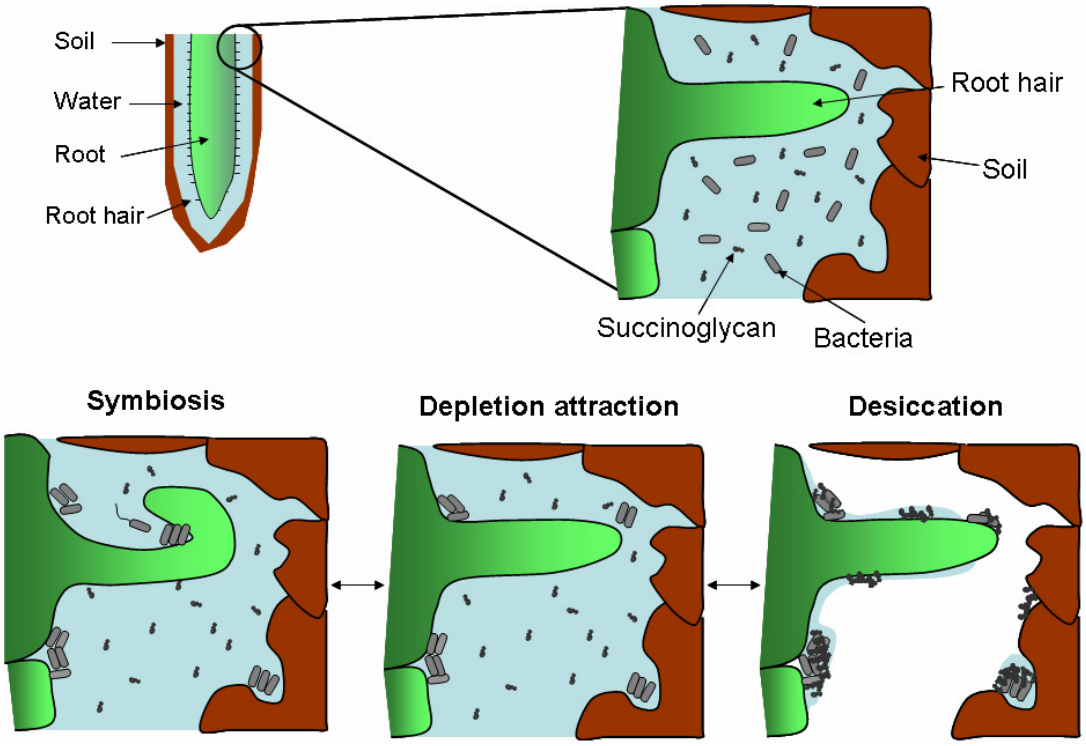


Figure 7-1. Model of aggregation of *S. meliloti* by succinoglycan in relation to its ecology when living in the rhizosphere. The rhizosphere is composed of microniches around the root hairs. In this relatively carbon-rich environment *S. meliloti* will produce succinoglycan resulting in aggregation of the cells by depletion attraction. The aggregates may then sediment onto surfaces or depletion attraction may also drive the attachment of cells to the root hair. Under low nitrogen conditions entry into the symbiosis with the plant host will be possible, with the upregulation of motility of the cells allowing them to leave the aggregates and move to colonise curled root hairs. Alternatively under desiccative conditions, evaporation of the water from the microniche will result in deposition of the cells onto the surface, with the concurrent deposition of succinoglycan facilitating attachment.

References

1. Costerton, J. W.; Cheng, K. J.; Geesey, G. G.; Ladd, T. I.; Nickel, J. C.; Dasgupta, M.; Marrie, T. J., Bacterial Biofilms in Nature and Disease. *Annual Review of Microbiology* 1987, 41, (1), 435-464.
2. Scragg, A., *Environmental Microbiology*. Longman: 1999.
3. Urbain, V.; Block, J. C.; Manem, J., Bioflocculation in Activated-Sludge - an Analytic Approach. *Water Research* 1993, 27, (5), 829-838.
4. McKinney, R. E., A Fundamental Approach to the Activated Sludge Process .2. a Proposed Theory of Floc Formation. *Sewage and Industrial Wastes* 1952, 24, (3), 280-287.
5. Tenney, M. W.; Stumm, W., Chemical Flocculation of Microorganisms in Biological Waste Treatment. *Journal (Water Pollution Control Federation)* 1965, 37, (10), 1370-1388.
6. Buchanan, R. E., AGGLUTINATION. *J. Bacteriol.* 1919, 4, (2), 73-105.
7. Harris, R. H.; Mitchell, R., Role of Polymers in Microbial Aggregation. *Annual Review of Microbiology* 1973, 27, 27-50.
8. Pavoni, J. L.; Echelber.Wf; Tenney, M. W., Bacterial Exocellular Polymers and Biological Flocculation. *Journal Water Pollution Control Federation* 1972, 44, (3), 414-420.
9. Deinema, M. H.; Zevenhuizen.Lp, Formation of Cellulose Fibrils by Gram-Negative Bacteria and Their Role in Bacterial Flocculation. *Archiv Fur Mikrobiologie* 1971, 78, (1), 42-51.
10. Friedman, B. A.; Dugan, P. R., Identification of *Zoogloea* species and the Relationship to Zoogloeal Matrix and Floc Formation. *J. Bacteriol.* 1968, 95, (5), 1903-1909.
11. Friedman, B. A.; Dugan, P. R.; Pfister, R. M.; Remsen, C. C., Structure of Exocellular Polymers and Their Relationship to Bacterial Flocculation. *J. Bacteriol.* 1969, 98, (3), 1328-1334.
12. McKinney, R. E., Staining bacterial polysaccharides. *J. Bacteriol.* 1953, 66, (4), 453-454.
13. McKinney, R. E.; Weichlein, R. G., Isolation of Floc-Producing Bacteria from Activated Sludge. *Appl. Environ. Microbiol.* 1953, 1, (5), 259-261.
14. Smit, G.; Kijne, J. W.; Lugtenberg, B. J. J., Correlation between Extracellular Fibrils and Attachment of *Rhizobium leguminosarum* to Pea Root Hair Tips. *Journal of Bacteriology* 1986, 168, (2), 821-827.
15. Smit, G.; Kijne, J. W.; Lugtenberg, B. J. J., Involvement of Both Cellulose Fibrils and a Ca-2+-Dependent Adhesin in the Attachment of *Rhizobium leguminosarum* to Pea Root Hair Tips. *Journal of Bacteriology* 1987, 169, (9), 4294-4301.
16. Matthysse, A. G., Role of bacterial cellulose fibrils in *Agrobacterium tumefaciens* infection. *J. Bacteriol.* 1983, 154, (2), 906-915.
17. Matthysse, A. G., Characterization of Nonattaching Mutants of *Agrobacterium tumefaciens*. *Journal of Bacteriology* 1987, 169, (1), 313-323.
18. Matthysse, A. G.; Marry, M.; Krall, L.; Kaye, M.; Ramey, B. E.; Fuqua, C.; White, A. R., The effect of cellulose overproduction on binding and biofilm

formation on roots by *Agrobacterium tumefaciens*. *Mol Plant Microbe Interact* 2005, 18, (9), 1002-10.
19. Sadasivan, L.; Neyra, C. A., Flocculation in *Azospirillum brasilense* and *Azospirillum lipoferum*: exopolysaccharides and cyst formation. *J. Bacteriol.* 1985, 163, (2), 716-723.
20. Del Gallo, M.; Negi, M.; Neyra, C. A., Calcofluor- and lectin-binding exocellular polysaccharides of *Azospirillum brasilense* and *Azospirillum lipoferum*. *J. Bacteriol.* 1989, 171, (6), 3504-3510.
21. Dow, J. M.; Crossman, L.; Findlay, K.; He, Y. Q.; Feng, J. X.; Tang, J. L., Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proceedings of the National Academy of Sciences of the United States of America* 2003, 100, (19), 10995-11000.
22. Torres, P. S.; Malamud, F.; Rigano, L. A.; Russo, D. M.; Marano, M. R.; Castagnaro, A. P.; Zorreguieta, A.; Bouarab, K.; Dow, J. M.; Vojnov, A. A., Controlled synthesis of the DSF cell-cell signal is required for biofilm formation and virulence in *Xanthomonas campestris*. *Environmental Microbiology* 2007, 9, 2101-2109.
23. Monier, J. M.; Lindow, S. E., Differential survival of solitary and aggregated bacterial cells promotes aggregate formation on leaf surfaces. *Proceedings of the National Academy of Sciences of the United States of America* 2003, 100, (26), 15977-15982.
24. Govan, J. R. W.; Deretic, V., Microbial pathogenesis in cystic fibrosis: Mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiological Reviews* 1996, 60, (3), 539-&.
25. Singh, P. K.; Schaefer, A. L.; Parsek, M. R.; Moninger, T. O.; Welsh, M. J.; Greenberg, E. P., Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 2000, 407, (6805), 762-764.
26. Costerton, J. W.; Stewart, P. S.; Greenberg, E. P., Bacterial biofilms: a common cause of persistent infections. *Science* 1999, 284, (5418), 1318-22.
27. Costerton, J. W.; Lewandowski, Z.; Caldwell, D. E.; Korber, D. R.; Lappinscott, H. M., Microbial Biofilms. *Annual Review of Microbiology* 1995, 49, 711-745.
28. Davey, M. E.; O'Toole, G. A., Microbial Biofilms: from Ecology to Molecular Genetics. *Microbiol. Mol. Biol. Rev.* 2000, 64, (4), 847-867.
29. O'Toole, G. A.; Kolter, R., Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. *Molecular Microbiology* 1998, 30, (2), 295-304.
30. O'Toole, G. A.; Pratt, L. A.; Watnick, P. I.; Newman, D. K.; Weaver, V. B.; Kolter, R., Genetic approaches to study of biofilms. *Biofilms* 1999, 310, 91-109.
31. Danese, P. N.; Pratt, L. A.; Kolter, R., Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. *J Bacteriol* 2000, 182, (12), 3593-6.
32. Madigan, M. T., Martino, J. M., *Brock Biology of Microorganisms*. 11th ed.; Pearson: 2006.
33. Becker, A.; Fraysse, N.; Sharypova, L., Recent advances in studies on structure and symbiosis-related function of rhizobial K-antigens and

lipopolysaccharides. *Molecular Plant-Microbe Interactions* 2005, 18, (9), 899-905.
34. Ulmer, A. J.; Rietschel, E. T.; Zahringer, U.; Heine, H., Lipopolysaccharide: Structure, bioactivity, receptors, and signal transduction. *Trends in Glycoscience and Glycotechnology* 2002, 14, (76), 53-68.
35. Whitfield, C., Bacterial Extracellular Polysaccharides. *Canadian Journal of Microbiology* 1988, 34, (4), 415-420.
36. Roberts, I. S., The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annual Review of Microbiology* 1996, 50, (1), 285-315.
37. Sutherland, I. W., *Biotechnology of microbial exopolysaccharides*. Cambridge University Press: Cambridge, 1990.
38. Wingender, J.; Neu, T. R.; Flemming, H.-C., *What are Bacterial Extracellular Polymeric Substances?* Springer: 1999; p 1-15.
39. Aspinall, G. O., *Isolation and fractionation of polysaccharides*. London, 1982; Vol. 1, p 19-34.
40. Sutherland, I. W., Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* 2001, 147, (1), 3-9.
41. Mendrygal, K. E.; Gonzalez, J. E., Environmental regulation of exopolysaccharide production in *Sinorhizobium meliloti*. *J Bacteriol* 2000, 182, (3), 599-606.
42. Sutherland, I. W., Structure-Function-Relationships in Microbial Exopolysaccharides. *Biotechnology Advances* 1994, 12, (2), 393-448.
43. Stoddart, R. W., *The biosynthesis of polysaccharides*. Macmillan Pub. Co.: New York, 1984.
44. Rees, D. A., *Polysaccharide shapes*. Chapman and Hall: London, 1977; p 77.
45. Rees, D. A., Morris, E. R., Thom, D., Madden, J. K., Shapes and Interactions of Carbohydrate Chains. In *The Polysaccharides*, Aspinall, G. O., Ed. Academic Press Inc.: London, 1982; Vol. 1, pp 196-292.
46. Deborah, M. R.; Daniel, J. W., Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. *Molecular Microbiology* 2005, 56, (2), 309-322.
47. Sriramulu, D. D.; Lunsdorf, H.; Lam, J. S.; Romling, U., Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. *Journal of Medical Microbiology* 2005, 54, (7), 667-676.
48. Ryder, C.; Byrd, M.; Wozniak, D. J., Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Current Opinion in Microbiology* 2007, 10, (6), 644-648.
49. Goldberg, J. B.; Ohman, D. E., Cloning and Expression in *Pseudomonas-aeruginosa* of a Gene Involved in the Production of Alginate. *Journal of Bacteriology* 1984, 158, (3), 1115-1121.
50. Linker, A.; Jones, R. S., A New Polysaccharide Resembling Alginic Acid Isolated from Pseudomonads. *J. Biol. Chem.* 1966, 241, (16), 3845-3851.
51. Jain, S.; Franklin, M. J.; Ertesvag, H.; Valla, S.; Ohman, D. E., The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. *Molecular Microbiology* 2003, 47, (4), 1123-1133.
52. Remminghorst, U.; Rehm, B. H. A., Bacterial alginates: from biosynthesis to applications. *Biotechnology Letters* 2006, 28, (21), 1701-1712.

53. Sutherland, I. W., Exopolysaccharides in biofilms, flocs and related structures. *Water Sci Technol* 2001, 43, (6), 77-86.
54. Wozniak, D. J.; Wyckoff, T. J. O.; Starkey, M.; Keyser, R.; Azadi, P.; O'Toole, G. A.; Parsek, M. R., Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proceedings of the National Academy of Sciences of the United States of America* 2003, 100, (13), 7907-7912.
55. Becker, A.; Katzen, F.; Puhler, A.; Ielpi, L., Xanthan gum biosynthesis and application: a biochemical/genetic perspective. *Applied Microbiology and Biotechnology* 1998, 50, (2), 145-152.
56. Jansson, P. E.; Kenne, L.; Lindberg, B., Structure of Extracellular Polysaccharide from *Xanthomonas campestris*. *Carbohydrate Research* 1975, 45, (DEC), 275-282.
57. Fujishige, N. A.; Kapadia, N. N.; Hirsch, A. M., A feeling for the micro-organism: structure on a small scale. Biofilms on plant roots. *Botanical Journal of the Linnean Society* 2006, 150, (1), 79-88.
58. Jones, K. M.; Kobayashi, H.; Davies, B. W.; Taga, M. E.; Walker, G. C., How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nat Rev Micro* 2007, 5, (8), 619-633.
59. Finan, T. M.; Hirsch, A. M.; Leigh, J. A.; Johansen, E.; Kuldau, G. A.; Deegan, S.; Walker, G. C.; Signer, E. R., Symbiotic mutants of rhizobium meliloti that uncouple plant from bacterial differentiation. *Cell* 1985, 40, (4), 869-877.
60. Leigh, J. A.; Signer, E. R.; Walker, G. C., Exopolysaccharide-Deficient Mutants of *Rhizobium meliloti* That Form Ineffective Nodules. *Proceedings of the National Academy of Sciences of the United States of America* 1985, 82, (18), 6231-6235.
61. Meade, H. M.; Long, S. R.; Ruvkun, G. B.; Brown, S. E.; Ausubel, F. M., Physical and Genetic-Characterization of Symbiotic and Auxotrophic Mutants of *Rhizobium meliloti* Induced by Transposon Tn5 Mutagenesis. *Journal of Bacteriology* 1982, 149, (1), 114-122.
62. Galibert, F.; Finan, T. M.; Long, S. R.; Puhler, A.; Abola, P.; Ampe, F.; Barloy-Hubler, F.; Barnett, M. J.; Becker, A.; Boistard, P.; Bothe, G.; Boutry, M.; Bowser, L.; Buhrmester, J.; Cadieu, E.; Capela, D.; Chain, P.; Cowie, A.; Davis, R. W.; Dreano, S.; Federspiel, N. A.; Fisher, R. F.; Gloux, S.; Godrie, T.; Goffeau, A.; Golding, B.; Gouzy, J.; Gurjal, M.; Hernandez-Lucas, I.; Hong, A.; Huizar, L.; Hyman, R. W.; Jones, T.; Kahn, D.; Kahn, M. L.; Kalman, S.; Keating, D. H.; Kiss, E.; Komp, C.; Lalaure, V.; Masuy, D.; Palm, C.; Peck, M. C.; Pohl, T. M.; Portetelle, D.; Purnelle, B.; Ramsperger, U.; Surzycki, R.; Thebault, P.; Vandenbol, M.; Vorholter, F. J.; Weidner, S.; Wells, D. H.; Wong, K.; Yeh, K. C.; Batut, J., The composite genome of the legume symbiont *Sinorhizobium meliloti. Science* 2001, 293, (5530), 668-672.
63. Sharypova, L. A.; Niehaus, K.; Scheidle, H.; Holst, O.; Becker, A., *Sinorhizobium meliloti* acpXL mutant lacks the C28 hydroxylated fatty acid moiety of lipid A and does not express a slow migrating form of lipopolysaccharide. *Journal of Biological Chemistry* 2003, 278, (15), 12946-12954.

64. Reuhs, B. L.; Geller, D. P.; Kim, J. S.; Fox, J. E.; Kolli, V. S. K.; Pueppke, S. G., *Sinorhizobium fredii* and *Sinorhizobium meliloti* Produce Structurally Conserved Lipopolysaccharides and Strain-Specific K Antigens. *Appl. Environ. Microbiol.* 1998, 64, (12), 4930-4938.
65. Fraysse, N.; Lindner, B.; Kaczynski, Z.; Sharypova, L.; Holst, O.; Niehaus, K.; Poinsot, V., *Sinorhizobium meliloti* strain 1021 produces a low-molecular-mass capsular polysaccharide that is a homopolymer of 3-deoxy-D-manno-oct-2-ulosonic acid harboring a phospholipid anchor. *Glycobiology* 2005, 15, (1), 101-108.
66. Sharypova, L. A.; Chataigne, G.; Fraysse, N.; Becker, A.; Poinsot, V., Overproduction and increased molecular weight account for the symbiotic activity of the rkpZ-modified K polysaccharide from *Sinorhizobium meliloti* Rm1021. *Glycobiology* 2006, 16, (12), 1181-1193.
67. Pellock, B. J.; Cheng, H. P.; Walker, G. C., Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. *J Bacteriol* 2000, 182, (15), 4310-8.
68. Åman, P.; McNeil, M.; Franzén, L.-E.; Darvill, A. G.; Albersheim, P., Structural elucidation, using h.p.l.c.-m.s. and g.l.c.-m.s., of the acidic polysaccharide secreted by *rhizobium meliloti* strain 1021. *Carbohydrate Research* 1981, 95, (2), 263-282.
69. Battisti, L.; Lara, J. C.; Leigh, J. A., Specific Oligosaccharide Form of the *Rhizobium meliloti* Exopolysaccharide Promotes Nodule Invasion in Alfalfa. *Proceedings of the National Academy of Sciences of the United States of America* 1992, 89, (12), 5625-5629.
70. Wang, L. X.; Wang, Y.; Pellock, B.; Walker, G. C., Structural characterization of the symbiotically important low-molecular-weight succinoglycan of *Sinorhizobium meliloti*. *J Bacteriol* 1999, 181, (21), 6788-96.
71. Zevenhuizen, L.; Vanneerven, A. R. W., (1-]2)-Beta-D-Glucan and Acidic Oligosaccharides Produced by *Rhizobium meliloti*. *Carbohydrate Research* 1983, 118, (JUL), 127-134.
72. Balnois, E.; Stoll, S.; Wilkinson, K. J.; Buffle, J.; Rinaudo, M.; Milas, M., Conformations of succinoglycan as observed by atomic force microscopy. *Macromolecules* 2000, 33, (20), 7440-7447.
73. Ridout, M. J.; Brownsey, G. J.; York, G. M.; Walker, G. C.; Morris, V. J., Effect of o-acyl substituents on the functional behaviour of *Rhizobium meliloti* succinoglycan. *Int J Biol Macromol* 1997, 20, (1), 1-7.
74. Her, G. R.; Glazebrook, J.; Walker, G. C.; Reinhold, V. N., Structural Studies of a Novel Exopolysaccharide Produced by a Mutant of *Rhizobium meliloti* Strain Rm1021. *Carbohydrate Research* 1990, 198, (2), 305-312.
75. Finan, T. M.; Kunkel, B.; Devos, G. F.; Signer, E. R., 2nd Symbiotic Megaplasmid in *Rhizobium meliloti* Carrying Exopolysaccharide and Thiamine Synthesis Genes. *Journal of Bacteriology* 1986, 167, (1), 66-72.
76. Tolmasky, M. E.; Staneloni, R. J.; Leloir, L. F., Lipid-bound saccharides in *Rhizobium meliloti*. *J. Biol. Chem.* 1982, 257, (12), 6751-6757.
77. Reuber, T. L.; Walker, G. C., Biosynthesis of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*. *Cell* 1993, 74, (2), 269-80.

78. Uttaro, A. D.; Cangelosi, G. A.; Geremia, R. A.; Nester, E. W.; Ugalde, R. A., Biochemical-Characterization of Avirulent Exoc Mutants of *Agrobacterium tumefaciens*. *Journal of Bacteriology* 1990, 172, (3), 1640-1646.
79. Glucksmann, M. A.; Reuber, T. L.; Walker, G. C., Genes needed for the modification, polymerization, export, and processing of succinoglycan by *Rhizobium meliloti*: a model for succinoglycan biosynthesis. *J Bacteriol* 1993, 175, (21), 7045-55.
80. Buendia, A. M.; Enenkel, B.; Koplin, R.; Niehaus, K.; Arnold, W.; Puhler, A., The *Rhizobium meliloti* Exozl Exob Fragment of Megaplasmid-2 - ExoB Functions as a UDP-Glucose 4-Epimerase and ExoZ Shows Homology to Nodx of *Rhizobium leguminosarum* Biovar Viciae Strain Tom. *Molecular Microbiology* 1991, 5, (6), 1519-1530.
81. Reuber, T. L.; Walker, G. C., The acetyl substituent of succinoglycan is not necessary for alfalfa nodule invasion by *Rhizobium meliloti* Rm1021. *J Bacteriol* 1993, 175, (11), 3653-5.
82. Gonzalez, J. E.; Semino, C. E.; Wang, L. X.; Castellano-Torres, L. E.; Walker, G. C., Biosynthetic control of molecular weight in the polymerization of the octasaccharide subunits of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*. *Proc Natl Acad Sci U S A* 1998, 95, (23), 13477-82.
83. York, G. M.; Walker, G. C., The *Rhizobium meliloti exoK* gene and *prsD/prsE/exsH* genes are components of independent degradative pathways which contribute to production of low-molecular-weight succinoglycan. *Mol Microbiol* 1997, 25, (1), 117-34.
84. York, G. M.; Walker, G. C., The *Rhizobium meliloti* ExoK and ExsH glycanases specifically depolymerize nascent succinoglycan chains. *Proc Natl Acad Sci U S A* 1998, 95, (9), 4912-7.
85. York, G. M.; Walker, G. C., The succinyl and acetyl modifications of succinoglycan influence susceptibility of succinoglycan to cleavage by the *Rhizobium meliloti* glycanases ExoK and ExsH. *J Bacteriol* 1998, 180, (16), 4184-91.
86. Doherty, D.; Leigh, J. A.; Glazebrook, J.; Walker, G. C., *Rhizobium meliloti* mutants that overproduce the *R. meliloti* acidic calcofluor-binding exopolysaccharide. *J Bacteriol* 1988, 170, (9), 4249-56.
87. Breedveld, M. W.; Zevenhuizen, L.; Zehnder, A. J. B., Osmotically Induced Oligosaccharide and Polysaccharide Synthesis by *Rhizobium meliloti* Su-47. *Journal of General Microbiology* 1990, 136, 2511-2519.
88. Reed, J. W.; Capage, M.; Walker, G. C., *Rhizobium meliloti* e*xoG* and Exoj Mutations Affect the Exox-Exoy System for Modulation of Exopolysaccharide Production. *Journal of Bacteriology* 1991, 173, (12), 3776-3788.
89. Zhan, H. J.; Leigh, J. A., Two genes that regulate exopolysaccharide production in *Rhizobium meliloti*. *J Bacteriol* 1990, 172, (9), 5254-9.
90. Quester, I.; Becker, A., Four promoters subject to regulation by ExoR and PhoB direct transcription of the *Sinorhizobium meliloti exoYFQ* operon involved in the biosynthesis of succinoglycan. *Journal of Molecular Microbiology and Biotechnology* 2004, 7, (3), 115-132.

91. Reuber, T. L.; Long, S.; Walker, G. C., Regulation of *Rhizobium meliloti* Exo Genes in Free-Living Cells and in Planta Examined by Using Tnphoa Fusions. *Journal of Bacteriology* 1991, 173, (2), 426-434.
92. Cheng, H. P.; Walker, G. C., Succinoglycan production by *Rhizobium meliloti* is regulated through the ExoS-ChvI two-component regulatory system. *J Bacteriol* 1998, 180, (1), 20-6.
93. Yao, S. Y.; Luo, L.; Har, K. J.; Becker, A.; Ruberg, S.; Yu, G. Q.; Zhu, J. B.; Cheng, H. P., *Sinorhizobium meliloti* ExoR and ExoS proteins regulate both succinoglycan and flagellum production. *J Bacteriol* 2004, 186, (18), 6042-9.
94. Chen, E. J.; Sabio, E. A.; Long, S. R., The periplasmic regulator ExoR inhibits ExoS/ChvI two-component signalling in *Sinorhizobium meliloti*. *Molecular Microbiology* 2008, 69, (5), 1290-1303.
95. Wells, D. H.; Chen, E. J.; Fisher, R. F.; Long, S. R., ExoR is genetically coupled to the ExoS-ChvI two-component system and located in the periplasm of *Sinorhizobium meliloti*. *Mol Microbiol* 2007, 64, (3), 647-64.
96. Glazebrook, J.; Walker, G. C., A Novel Exopolysaccharide Can Function in Place of the Calcofluor-Binding Exopolysaccharide in Nodulation of Alfalfa by *Rhizobium meliloti*. *Cell* 1989, 56, (4), 661-672.
97. Pellock, B. J.; Teplitski, M.; Boinay, R. P.; Bauer, W. D.; Walker, G. C., A LuxR homolog controls production of symbiotically active extracellular polysaccharide II by *Sinorhizobium meliloti*. *J Bacteriol* 2002, 184, (18), 5067-76.
98. Russo, D. M.; Williams, A.; Edwards, A.; Posadas, D. M.; Finnie, C.; Dankert, M.; Downie, J. A.; Zorreguieta, A., Proteins exported via the PrsD-PrsE type I secretion system and the acidic exopolysaccharide are involved in biofilm formation by *Rhizobium leguminosarum*. *J Bacteriol* 2006, 188, (12), 4474-86.
99. Fujishige, N. A.; Lum, M. R.; De Hoff, P. L.; Whitelegge, J. P.; Faull, K. F.; Hirsch, A. M., Rhizobium common nod genes are required for biofilm formation. *Mol Microbiol* 2008, 67, (3), 504-15.
100. Liu, X. M.; Sheng, G. P.; Yu, H. Q., DLVO approach to the flocculability of a photosynthetic H2-producing bacterium, *Rhodopseudomonas acidophila*. *Environ Sci Technol* 2007, 41, (13), 4620-5.
101. Kurane, R.; Matsuyama, H., Production of a Bioflocculant by Mixed Culture. *Bioscience Biotechnology and Biochemistry* 1994, 58, (9), 1589-1594.
102. Busch, P. L.; Stumm, W., Chemical Interactions In the aggregation of bacteria bioflocculation in waste treatment. *Environ Sci Technol* 1968, 2, ((1)), 49-53.
103. Ries, H. E.; Meyers, B. L., Flocculation Mechanism - Charge Neutralization and Bridging. *Science* 1968, 160, (3835), 1449-&.
104. Adams, M.; Dogic, Z.; Keller, S. L.; Fraden, S., Entropically driven microphase transitions in mixtures of colloidal rods and spheres. *Nature* 1998, 393, (6683), 349-352.
105. Marenduzzo, D.; Finan, K.; Cook, P. R., The depletion attraction: an underappreciated force driving cellular organization. *J. Cell Biol.* 2006, 175, (5), 681-686.
106. Yodh, A. G.; Lin, K. H.; Crocker, J. C.; Dinsmore, A. D.; Verma, R.; Kaplan, P. D., Entropically driven self-assembly and interaction in suspension.

Philosophical Transactions of the Royal Society of London Series a-Mathematical Physical and Engineering Sciences 2001, 359, (1782), 921-937.
107. Tuinier, R.; de Kruif, C. G., Phase Separation, Creaming, and Network Formation of Oil-in-Water Emulsions Induced by an Exocellular Polysaccharide. *Journal of Colloid and Interface Science* 1999, 218, (1), 201-210.
108. Ellis, R. J., Macromolecular crowding: obvious but underappreciated. *Trends in Biochemical Sciences* 2001, 26, (10), 597-604.
109. Minton, A. P., The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *Journal of Biological Chemistry* 2001, 276, (14), 10577-10580.
110. Minton, A. P., Macromolecular crowding. *Current Biology* 2006, 16, (8), R269-R271.
111. Kim, E. H.; Chow, W. S.; Horton, P.; Anderson, J. M., Entropy-assisted stacking of thylakoid membranes. *Biochimica Et Biophysica Acta-Bioenergetics* 2005, 1708, (2), 187-195.
112. Zimmerman, S. B., Macromolecular Crowding Effects on Macromolecular Interactions - Some Implications for Genome Structure and Function. *Biochimica Et Biophysica Acta* 1993, 1216, (2), 175-185.
113. Zimmerman, S. B., Shape and compaction of *Escherichia coli* nucleoids. *Journal of Structural Biology* 2006, 156, (2), 255-261.
114. Parker, A.; Gunning, P. A.; Ng, K.; Robins, M. M., How does xanthan stabilise salad dressing? *Food Hydrocolloids* 1995, 9, (4), 333-342.
115. Cao, Y.; Dickinson, E.; Wedlock, D. J., Creaming and Flocculation in Emulsions Containing Polysaccharide. *Food Hydrocolloids* 1990, 4, (3), 185-196.
116. Tuinier, R.; ten Grotenhuis, E.; Holt, C.; Timmins, P. A.; de Kruif, C. G., Depletion interaction of casein micelles and an exocellular polysaccharide. *Physical Review E* 1999, 60, (1), 848.
117. Eboigbodin, K. E.; Newton, J. R.; Routh, A. F.; Biggs, C. A., Role of nonadsorbing polymers in bacterial aggregation. *Langmuir* 2005, 21, (26), 12315-9.
118. Liu, H. H.; Yang, Y. R.; Shen, X. C.; Zhang, Z. L.; Shen, P.; Xie, Z. X., Role of DNA in bacterial aggregation. *Current Microbiology* 2008, 57, (2), 139-144.
119. Leigh, J. A.; Reed, J. W.; Hanks, J. F.; Hirsch, A. M.; Walker, G. C., *Rhizobium meliloti* Mutants That Fail to Succinylate Their Calcofluor-Binding Exopolysaccharide Are Defective in Nodule Invasion. *Cell* 1987, 51, (4), 579-587.
120. Guyer, M. S.; Reed, R. R.; Steitz, J. A.; Low, K. B., Identification of a sex-factor-affinity site in *E. coli* as gamma delta. *Cold Spring Harb Symp Quant Biol* 1981, 45 Pt 1, 135-40.
121. Hanahan, D., Studies on Transformation of *Escherichia coli* with Plasmids. *Journal of Molecular Biology* 1983, 166, (4), 557-580.
122. Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, Y.; Baba, M.; Datsenko, K. A.; Tomita, M.; Wanner, B. L.; Mori, H., Construction of

Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology* 2006, 2.
123. Cheng, H. P.; Walker, G. C., Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. *J Bacteriol* 1998, 180, (19), 5183-91.
124. Sambrook, J., Fritsch, E. F., Maniatis, T., *Molecular Cloning: A laboratory Manual*. Cold Spring Harbor Laboratory Press: 1989; Vol. 3.
125. Rasband, W., http://rsb.info.nih.gov/ij. 2008.
126. Barthel, K. http://rsb.info.nih.gov/ij/plugins/volume-viewer.html.
127. Walker, T. S.; Bais, H. P.; Deziel, E.; Schweizer, H. P.; Rahme, L. G.; Fall, R.; Vivanco, J. M., *Pseudomonas aeruginosa*-plant root interactions. Pathogenicity, biofilm formation, and root exudation. *Plant Physiology* 2004, 134, (1), 320-331.
128. Glucksmann, M. A.; Reuber, T. L.; Walker, G. C., Family of glycosyl transferases needed for the synthesis of succinoglycan by *Rhizobium meliloti*. *J Bacteriol* 1993, 175, (21), 7033-44.
129. Reuber, T. L.; Urzainqui, A.; Glazebrook, J.; Reed, J. W.; Walker, G. C., Rhizobium meliloti exopolysaccharides. Structures, genetic analyses, and symbiotic roles. *Ann N Y Acad Sci* 1991, 646, 61-8.
130. Reuber, T. L.; Reed, J.; Glazebrook, J.; Glucksmann, M. A.; Ahmann, D.; Marra, A.; Walker, G. C., *Rhizobium meliloti* exopolysaccharides: genetic analyses and symbiotic importance. *Biochem Soc Trans* 1991, 19, (3), 636-41.
131. Glazebrook, J.; Reed, J. W.; Reuber, T. L.; Walker, G. C., Genetic analyses of *Rhizobium meliloti* exopolysaccharides. *Int J Biol Macromol* 1990, 12, (2), 67-70.
132. Fujishige, N. A.; Kapadia, N. N.; De Hoff, P. L.; Hirsch, A. M., Investigations of *Rhizobium* biofilm formation. *FEMS Microbiol Ecol* 2006, 56, (2), 195-206.
133. Eboigbodin, K. E.; Biggs, C. A., Characterization of the extracellular polymeric substances produced by *Escherichia coli* using infrared spectroscopic, proteomic, and aggregation studies. *Biomacromolecules* 2008, 9, (2), 686-95.
134. Eboigbodin, K. E.; Newton, J. R.; Routh, A. F.; Biggs, C. A., Bacterial quorum sensing and cell surface electrokinetic properties. *Appl Microbiol Biotechnol* 2006, 73, (3), 669-75.
135. Jucker, B. A.; Harms, H.; Zehnder, A. J., Adhesion of the positively charged bacterium *Stenotrophomonas* (*Xanthomonas*) *maltophilia* 70401 to glass and Teflon. *J. Bacteriol.* 1996, 178, (18), 5472-5479.
136. Biosciences, A., *Gel Filtration: Principles and methods*. 2002.
137. Nicholson, J. W., *The Chemistry of Polymers*. The Royal Society of Chemistry: Cambridge, 1991.
138. Nakanishi, T.; Norisuye, T., Thermally Induced Conformation Change of Succinoglycan in Aqueous Sodium Chloride. *Biomacromolecules* 2003, 4, (3), 736-742.
139. Neu, T. R.; Lawrence, J. R., Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy. *Fems Microbiology Ecology* 1997, 24, (1), 11-25.

140. Dentini, M.; Coviello, T.; Burchard, W.; Crescenzi, V., Solution Properties of Exocellular Microbial Polysaccharides .3. Light-Scattering from Gellan and from the Exocellular Polysaccharide of *Rhizobium trifolii* (Strain-Ta-1) in the Ordered State. *Macromolecules* 1988, 21, (11), 3312-3320.
141. Helden, L.; Roth, R.; Koenderink, G. H.; Leiderer, P.; Bechinger, C., Direct measurement of entropic forces induced by rigid rods. *Phys Rev Lett* 2003, 90, (4), 048301.
142. Koenderink, G. H.; Vliegenthart, G. A.; Kluijtmans, S.; van Blaaderen, A.; Philipse, A. P.; Lekkerkerker, H. N. W., Depletion-induced crystallization in colloidal rod-sphere mixtures. *Langmuir* 1999, 15, (14), 4693-4696.
143. Lin, K. H.; Crocker, J. C.; Zeri, A. C.; Yodh, A. G., Colloidal interactions in suspensions of rods (vol 87, art. no. 088301, 2001). *Physical Review Letters* 2001, 87, (26).
144. Nesper, J.; Hill, C. M. D.; Paiment, A.; Harauz, G.; Beis, K.; Naismith, J. H.; Whitfield, C., Translocation of group 1 capsular polysaccharide in *Escherichia coli* serotype K30 - Structural and functional analysis of the outer membrane lipoprotein Wza. *Journal of Biological Chemistry* 2003, 278, (50), 49763-49772.
145. Troy, F. A.; Frerman, F. E.; Heath, E. C., The Biosynthesis of Capsular Polysaccharide in Aerobacter aerogenes. *J. Biol. Chem.* 1971, 246, (1), 118-133.
146. Mayer, C.; Moritz, R.; Kirschner, C.; Borchard, W.; Maibaum, R.; Wingender, J.; Flemming, H.-C., The role of intermolecular interactions: studies on model systems for bacterial biofilms. *International Journal of Biological Macromolecules* 1999, 26, (1), 3-16.
147. Vuong, C.; Kocianova, S.; Voyich, J. M.; Yao, Y.; Fischer, E. R.; DeLeo, F. R.; Otto, M., A Crucial Role for Exopolysaccharide Modification in Bacterial Biofilm Formation, Immune Evasion, and Virulence. *J. Biol. Chem.* 2004, 279, (52), 54881-54886.
148. Schwarz-Linek, J.; Winkler, A.; Wilson, L. G.; Pham, N. T.; Schilling, T.; Poon, W. C. K., Phase separation in *Escherichia coli* suspensions induced by anionic polymers: a study by experiments and simulations. *In preparation* 2009.
149. Bachmann, B. J., Pedigrees of Some Mutant Strains of *Escherichia coli* K-12. *Bacteriological Reviews* 1972, 36, (4), 525-557.
150. Pesavento, C.; Becker, G.; Sommerfeldt, N.; Possling, A.; Tschowri, N.; Mehlis, A.; Hengge, R., Inverse regulatory coordination of motility and curli-mediated adhesion in *Escherichia coli*. *Genes & Development* 2008, 22, (17), 2434-2446.
151. Sharma, A.; Tan, S. N.; Walz, J. Y., Effect of nonadsorbing polyelectrolytes on colloidal interactions in aqueous mixtures. *Journal of Colloid and Interface Science* 1997, 191, (1), 236-246.
152. Shimada, T.; Makinoshima, H.; Ogawa, Y.; Miki, T.; Maeda, M.; Ishihama, A., Classification and strength measurement of stationary-phase promoters by use of a newly developed promoter cloning vector. *Journal of Bacteriology* 2004, 186, (21), 7112-7122.

153. Donlan, R. M.; Costerton, J. W., Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews* 2002, 15, (2), 167-+.
154. Kolter, R.; Losick, R., One for All and All for One. *Science* 1998, 280, (5361), 226-227.
155. Watnick, P.; Kolter, R., Biofilm, city of microbes. *Journal of Bacteriology* 2000, 182, (10), 2675-2679.
156. Danhorn, T.; Fuqua, C., Biofilm formation by plant-associated bacteria. *Annu Rev Microbiol* 2007, 61, 401-22.
157. Branda, S. S.; Gonzalez-Pastor, J. E.; Dervyn, E.; Ehrlich, S. D.; Losick, R.; Kolter, R., Genes involved in formation of structured multicellular communities by *Bacillus subtilis*. *Journal of Bacteriology* 2004, 186, (12), 3970-3979.
158. Terpolilli, J. J.; O'Hara, G. W.; Tiwari, R. P.; Dilworth, M. J.; Howieson, J. G., The model legume *Medicago truncatula* A17 is poorly matched for N-2 fixation with the sequenced microsymbiont *Sinorhizobium meliloti* 1021. *New Phytologist* 2008, 179, (1), 62-66.
159. Roberson, E. B.; Firestone, M. K., Relationship between Desiccation and Exopolysaccharide Production in a Soil *Pseudomonas sp*. *Appl. Environ. Microbiol.* 1992, 58, (4), 1284-1291.
160. Schnider-Keel, U.; Lejbolle, K. B.; Baehler, E.; Haas, D.; Keel, C., The Sigma Factor AlgU (AlgT) Controls Exopolysaccharide Production and Tolerance towards Desiccation and Osmotic Stress in the Biocontrol Agent *Pseudomonas fluorescens* CHA0. *Appl. Environ. Microbiol.* 2001, 67, (12), 5683-5693.
161. Chang, W.-S.; van de Mortel, M.; Nielsen, L.; Nino de Guzman, G.; Li, X.; Halverson, L. J., Alginate Production by *Pseudomonas putida* Creates a Hydrated Microenvironment and Contributes to Biofilm Architecture and Stress Tolerance under Water-Limiting Conditions. *J. Bacteriol.* 2007, 189, (22), 8290-8299.
162. Ausmees, N.; Jacobsson, K.; Lindberg, M., A unipolarly located, cell-surface-associated agglutinin, RapA, belongs to a family of Rhizobium-adhering proteins (Rap) in *Rhizobium leguminosarum* bv. *trifolii*. *Microbiology* 2001, 147, (3), 549-559.
163. Laus, M. C.; Logman, T. J.; Lamers, G. E.; Van Brussel, A. A. N.; Carlson, R. W.; Kijne, J. W., A novel polar surface polysaccharide from *Rhizobium leguminosarum* binds host plant lectin. *Molecular Microbiology* 2006, 59, (6), 1704-1713.
164. Williams, A.; Wilkinson, A.; Krehenbrink, M.; Russo, D. M.; Zorreguieta, A.; Downie, J. A., Glucomannan-Mediated Attachment of *Rhizobium leguminosarum* to Pea Root Hairs Is Required for Competitive Nodule Infection. *J. Bacteriol.* 2008, 190, (13), 4706-4715.
165. Merker, R. I.; Smit, J., Characterization of the Adhesive Holdfast of Marine and Freshwater Caulobacters. *Appl. Environ. Microbiol.* 1988, 54, (8), 2078-2085.
166. Goodman, A. L.; Kulasekara, B.; Rietsch, A.; Boyd, D.; Smith, R. S.; Lory, S., A signaling network reciprocally regulates genes associated with acute

infection and chronic persistence in *Pseudomonas aeruginosa*. *Developmental Cell* 2004, 7, (5), 745-754.
167. O'Toole, G. A., Microbiology - Jekyll or hide? *Nature* 2004, 432, (7018), 680-681.